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Impact of energy restriction during late gestation on muscle and blood transcriptome of beef calves

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Impact of energy restriction during late gestation on muscle and blood transcriptome of beef calves

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To Nick, Moysés, and Márcio, for being outstanding mentors and source of inspiration. To my mother, Dayse, for the encouragement throughout all my life. To my sisters and best friends, Lilian and Arabela, for being so supportive even being far away. To my boyfriend, Victor, for the patience, love, and friendship. To my grandparents, Ada and Magela, for supporting me in my determination to find and pursuit my goals; this is for you!

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NOMENCLATURE

ACTH	Adrenocorticoptropin hormone
AIC	Akaike information criterion
ATP6V0D1	ATPase H+ transporting V0 subunit d1
Bp	Base pairs
C/EBP	CCAAT-enhancer-binding proteins
CABLES1	Cdk5 and Abl enzyme substrate 1
COL1A1	Type I collagen
CRF	Corticotropin releasing factor
CTRL	Control group
DEG	Differentially expressed genes
DF	Degree-of-freedom
DPW	Days post weaning
EIF2S3	Eukaryotic translation initiation factor 2 subunit gamma
ETFRF1	Electron transfer flavoprotein regulatory factor 1
ETNPPL	Ethanolamine-phosphate phospholyase
FC	Fold change
FDR	False-discovery rate
FN	Fibronectin
FZD5	Frizzled class receptor 5
GCGR	Glucagon receptor
GO	Gene Ontology
GPC4	Glypican 4

GTF	Gene transfer format
GTF2A1L	General transcription factor IIA subunit 1 like
H19	H19, Imprinted Maternally Expressed Transcript
HPA	Hypothalamic-pituitary-adrenal
ICMT	Isoprenylcysteine carboxyl methyltransferase
IFNγ	Interferon gamma
IGF2R	Insulin-like growth factor 2 receptor
IL	Interleukin
ILF3	Interleukin enhancer binding factor 3
INO80D	INO80 complex subunit D
KDM6A	Lysine demethylase 6A
KLRF1	Killer cell lectin like receptor F1
LOC104968634	Antimicrobial peptide NK-lysin-like
LOC107131189	Eukaryotic translation initiation factor 2 subunit 3, Y-linked-like
LOC107131247	Multidrug resistance-associated protein 4-like
LOC512150	Myeloid-associated differentiation marker-like
LTR	Long terminal repeat
MATK	Megakaryocyte-associated tyrosine kinase
Med	Median
mTOR	Protein kinase B/rapamycin
MPSS	Massively Parallel Signature Sequencing
MRF	Myogenic regulator factors
MYF5	Myogenic factor 5

MYOD1	Myogenic Differentiation 1
MYOG	Myogenin
NB	Negative binomial
NK	Natural killer
OASL	2'-5'-oligoadenylate synthetase like
Pax	Paired box
PcG	Polycomb group proteins
PD-1	Programmed cell death 1 ligand 1
PE	Paired-end
PPARγ	Peroxisome proliferator-activated receptor gamma
Pref-1	Preadipocyte factor-1
PUFA	Poly-unsaturated fatty acid
QDPR	Quinoid dihydropteridine reductase
qPCR	Quantitative PCR
RASD2	RASD family member 2
REST	Energy-restricted group
RIMS1	Regulating synaptic membrane exocytosis 1
RIN	RNA integrity number
RORα	Retinoid acid receptor-related orphan receptor alpha
RNA-seq	RNA sequencing method
RPKM	Reads Per Kilobase per Million
SAGE	Serial Analysis of Gene Expression
SE	Single-end

SHH	Sonic hedgehog
SIM	Simple or uncorrelated structure
SLC20A2	Solute carrier family 20 member 2
SLCO3A1	Solute carrier organic anion transporter family member 3A1
SNP	Single nucleotide polymorphisms
SPAG17	Sperm associated antigen 17
SYAP1	Synapse associated protein 1
SYT3	Synaptotagmin 3
TGF	Transforming growth factor
TMCC2	Transmembrane and coiled-coil domain family 2
TMM	Trimmed Mean of M-values
TrxG	Trithorax
UQ	Upper Quartile
USF3	Upstream transcription factor family member 3
USP9X	Ubiquitin specific peptidase 9, X-linked
VAT1	Vesicle amine transport 1

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ABSTRACT

Maternal nutrition during gestation has long-term effects on skeletal muscle development of the offspring. In this thesis, we studied the effect of energy restriction during late gestation on gene expression in the blood and muscle tissues of preconditioned beef calves. The first chapter corresponds to a literature review in which we describe aspects related to this research, such as muscle and immune system development, gene expression, and the use of RNA-seq data to answer complex biological questions. The second chapter describes a research project in which, multiparous cows were divided into two groups: cows fed 70% and 100% of their energy requirements during the last third of gestation. Blood and muscle samples were collected from the offspring, and total RNA was extracted and sequenced (RNA-seq). Finally, statistical analyses were performed to identify differentially expressed genes as an effect of maternal energy restriction, overrepresentation of related biological functions, and to construct gene-gene networks. A total of 160, 164, and 346 DEG (q-value < 0.05) were identified in the skeletal muscle for the effects of diet, sex, and diet-by-sex interaction, respectively. For blood, 452, 1392, and 155 DEG were identified for the effects of diet, time, and diet-by-time interaction, respectively. For skeletal muscle, results based on diet identified genes involved in the muscle metabolism. On muscle, from the 10 most DEG down-regulated in the energy-restricted group (REST), we identified 6 genes associated with muscle metabolism and development, *SLCO3A1*, *ATP6V0D1*, *SLC2A1*, *GPC4*, *RASD2*, and *SLC2A1*. On blood, among the top 10 DEG, we found genes related to response to stress overexpressed in the REST after weaning, such as *SOD3* and *INO80D*, and to immune response down-regulated in the REST after vaccination, such as *OASL,*

KLRF1, and *LOC104968634.* Among the DEG identified in both blood and muscle, including *VAT1, CABLES1, SLC20A2, ILF3,* and *QDPR* were down-regulated in the muscle and up-regulated in blood, and *SPAG17* and *LOC107131247* were down-regulated in both.

Therefore, the findings support the hypothesis that alterations in the intra-uterine environment can alter the gene expression of muscle and blood on the offspring which and, consequently, modify postnatal development.

CHAPTER 1. LITERATURE REVIEW

Introduction

The deposition of skeletal muscle has significant implications for animal meat-production systems. The number and size of muscle fibers are important to establish muscle mass. Muscle fibers are formed mainly during the mid-gestation, and the definitive number is determined before birth [1]. On the other hand, skeletal muscle fiber hypertrophy occurs in late gestation and continues in postnatal development [1]. In addition, the heath of the animals impacts performance independently of the category of the animal, since a well-developed immune system will protect the animal from pathogens [2,3]. Moreover, immune system programming could give rise to changes in the fetal immune system that persists over the course of life [4].

Maternal nutrition has been pointed as one of the main factors affecting intra-uterine environment, delaying or enhancing fetal development [5]. The increase in nutritional requirements by beef cows during late gestation [6], can cause lack of nutrients for optimum fetal development [7]. Metabolizable energy restriction has been shown to downregulate the mitogenic responses of T lymphocytes from calves [8]. Thus, prenatal programming of physiological systems can alter the growth and function of organ systems and pathology into adulthood [9]. In other words, nutritional deficiency may affect the expression of genes regulating myogenesis and immune response in the fetus, which can have long-term effects on progeny development affecting the productive performance [10], and, consequently, the livestock industry [5]. Therefore, the last trimester of gestation is a critical period for fetal growth [10].

The increase in size of muscle fibers continues after birth, making this phase also critical for proper muscle development. Preconditioning phase is a crucial period for beef calves, since the animals pass through stressful procedures such as vaccination, weaning, and diet changes [11,12]. As a response, there is a mobilization of protein from muscle [13] to increase the synthesis of proteins and cells involved in the immune system [14] . Consequently, the growth performance is compromised due to limited availability of nutrients to muscle development. Also during this period, there is a decrease in energy and protein intake [3] which can reduce innate immune system and, consequently, impact vaccination response [12].

Sequencing-based methods have been widely used to identify differentially expressed genes and pathways in animals under different biological conditions. Among these technologies, RNA-seq has several advantages, including whole transcriptome analysis, sensitivity for lowly expressed transcript quantification, and detection of expressed genes involved in specific functions (i.e., muscle development and immune response) [15].

Fetal Development

Muscle Development

Fetal muscle development and myogenesis

The fetal stage is especially important for skeletal muscle development because the muscle fibers are formed mainly during mid-gestation period, during 2 and 7 months in beef cattle [1], and the definitive number is determined before birth [16]. Skeletal muscle is composed of muscle fibers, fat cells, and connective tissue, all derived from mesenchymal multipotent cells; therefore, skeletal muscle development involves myogenesis, adipogenesis, and fibrogenesis [17].

The development of skeletal muscle initiates during the embryonic stage [18]. The activation of *Wnt pathway signaling* and *SHH* works as signals to commit portion of the mesenchymal stem cells to the myogenic lineage [19]. This stimulation regulates the expression of *paired box (Pax) 3*, *Pax7*, and *GLI1* [20]. First, Pax3 and Pax7 are expressed in the mesoderm, which acts upon two myogenic regulator factors (MRF), *MYF-5*, and *MYOD-1* [20]. As a result, the proliferating myoblasts withdrawal from cell cycle to start to differentiate. Concomitantly, myoblasts initiate the expression of another important MRF, *MYOG*, which is necessary for the formation of multinucleated myotubes. Primary myofibers are first formed in the embryonic stage, followed by the formation of secondary myofibers in the mid and late gestation in humans, and late and neonatal stages in mice [21]. The formation of secondary myofibers overlaps with adipogenesis which is initiated at mid gestation in humans and late gestation in rodents [16].

Adipogenesis is initiated around mid-gestation and peaks near parturition (Figure 1.1) [21]. The mechanisms controlling adipogenesis have been primarily studied *in vitro* by cell culture studies [22]. Adipogenesis is mainly controlled by Peroxisome proliferator-activated receptor gamma (*PPARγ*) and *CCAAT-enhancer-binding proteins* (*C/EBP*) [23]. The activation of *PPARγ* stimulates the transcription factors, *C/EBPβ* and *C/EBPδ*. Also, it stimulates *C/EBPα*, which promotes the differentiate phenotype [23].

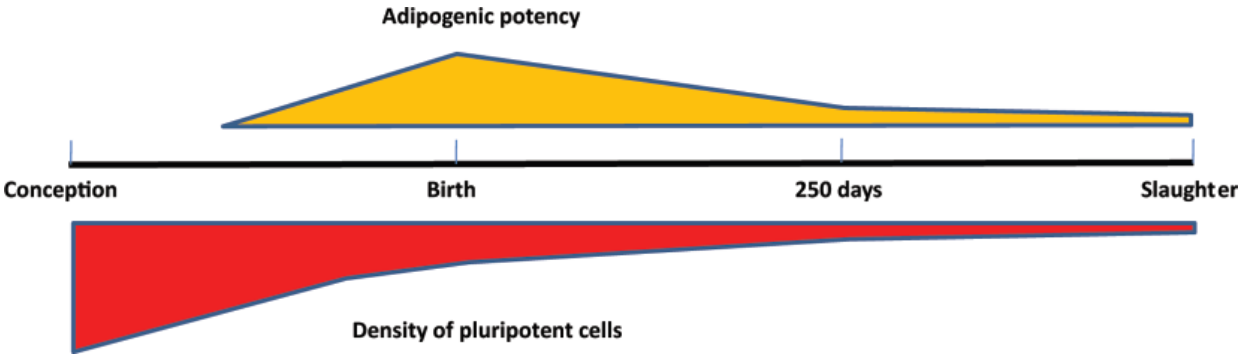

Figure 1.1 *Density of multipotent cells and adipogenic potency of bovine skeletal muscle. The dates are approximate and represent the progression through the various developmental stages. Adipogenesis is initiated around mid-gestation in ruminant animals and peaks near the birth. The adipogenic potency gradually declines postnatally because of depletion of pluripotent cells* [16].

Fibrogenesis in fetal skeletal muscle occurs mainly in mid gestation, coinciding with adipogenesis, although it seems to be ongoing during the whole gestation. This process is mainly regulated by Bone Morphogenic Proteins (BMPs), which belongs to the Transforming growth factor (*TGF) alpha (TGF-α*) super family. The regulatory *Smads*, *Smad 1*, *5* and/or *8*, bind to the BMPs becoming activated, which associates with Smad 4 to enhance adipogenesis. Then this complex is translocated into the nucleus to initiate transcription of *TGF-α* target genes [24,25], including *FN* and *COL1A1* [26].

Together, these three mechanisms - myogenesis, adipogenesis, fibrogenesis - produce the basic structure of skeletal muscle. Because most myocytes, adipocytes, and fibroblasts in the fetal muscle are derived from the same pool of mesenchymal stem cells, defining the mechanisms regulating mesenchymal stem cell differentiation in fetal muscle is essential to improving animal production efficiency [17].

Effect of prenatal muscle growth on performance

Alterations during fetal stage of the offspring can affect the offspring performance quantitatively and qualitatively (Figure 1.2). Since most of muscle fibers are formed during the fetal stage, limited number of fibers developed during this stage can negatively affect the physiological functions of the offspring, with consequences to carcass yield and production efficiency [27,28].

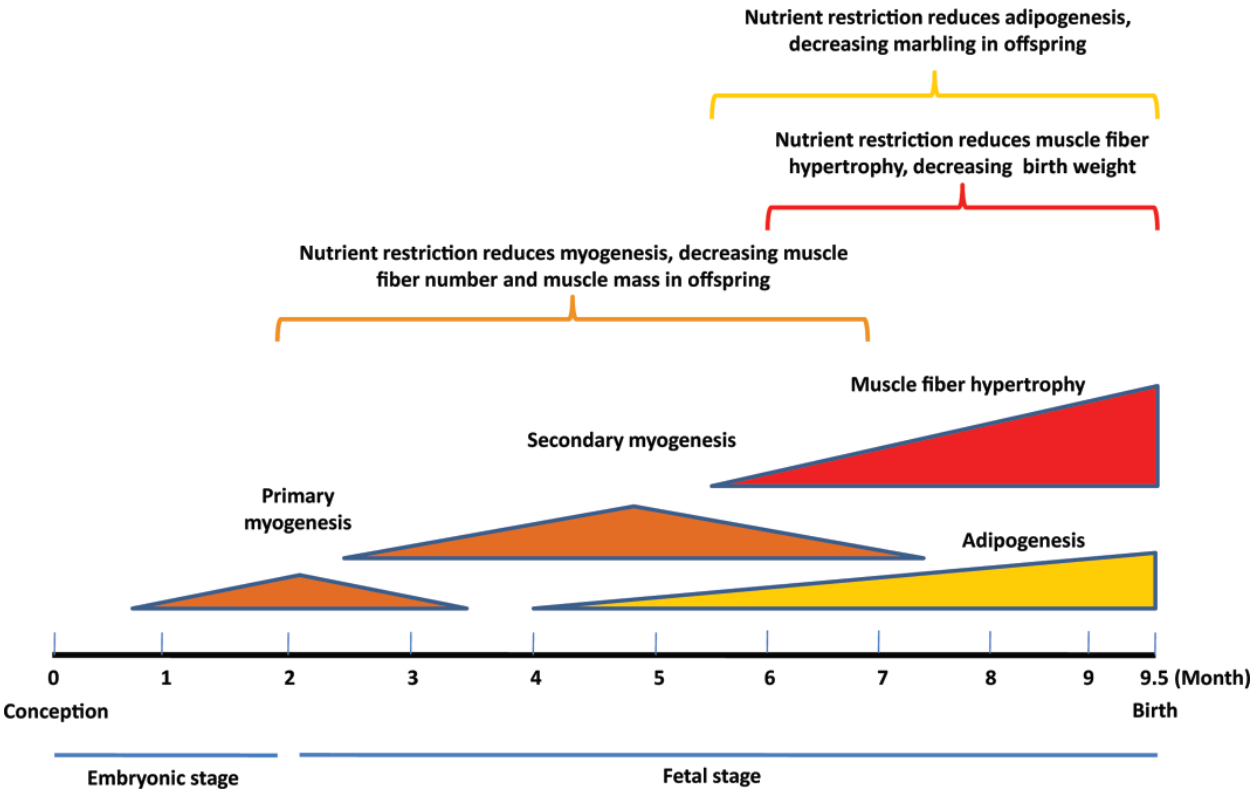

Figure 1.2 *Effects of maternal nutrition on bovine fetal skeletal muscle development. The dates are estimated mainly based on data from studies in sheep, rodents, and humans and represent the progression through the various developmental stages. Nutrient restriction during mid-gestation reduces muscle fiber numbers, whereas restriction during late gestation reduces both muscle fiber sizes and the formation of intramuscular adipocytes* [16].

The amount of intramuscular fat (i.e., marbling), which contributes to both meat flavor and texture, is determined by the number and size of intramuscular adipocytes. As discussed previously, both secondary muscle fibers and adipocytes are formed mainly during

mid gestation and are derived from the same pool of mesenchymal stem cells, generating a competition between them [29]. Therefore, any management alterations, such as changing maternal nutrition which enhances the number of mesenchymal cells committed to adipogenesis, will increase the number of intramuscular adipocytes and thus marbling [16]. This stage of fetal development is also associated with fibrogenesis, which generates the connective tissue [17]. This tissue is necessary for muscle development since it is part of the extracellular matrix; however, if in excess it is associated with toughness of the meat and, therefore, is not desirable [17].

Adipogenesis and fibrogenesis are as important as myogenesis for proper muscle development. Therefore, studies that aim to understand their generation and development in fetal skeletal muscle are imperative.

Muscle fiber types

Skeletal muscle is formed by muscle fibers, which can be classified as being red or white, basically based on their color intensity [30]. Also, the muscle fiber types differ about the speed of contraction and the type of energy metabolism in each type of fiber [31].

The primary function of the muscle is to contract and to create tension between two anatomical locations, and the function can be adjusted according to the needs of the animal [32]. One of the main proteins controlling the contraction is myosin whose type define the speed of contraction. Basically, myosin binds to actin to activate the ATPase activity and to promote the contraction [33]. Therefore, the classification of muscle fibers is based on the actomyosin ATPase activity, in which the fibers can be classified in type 1, 2A, and 2B [31].

In addition to the type of myosin and speed of contraction, muscle fibers can be classified based on the type of enzymes used to supply energy for contraction, glycolytic or

oxidative. Glycolytic metabolism provides an abundance of lactate dehydrogenase whereas oxidative provides succinate dehydrogenase, adenine dinucleotide, and tetrazolium reductase [34]. Adding the energy metabolism compound to the myosin type classification, add a new class of fiber type. So, now the fibers can be classified as type 1, 2A, 2X, and 2B [35].

Along with this classification, muscle fibers types differ in many characteristics, such as content of myoglobin, fiber diameter, fatigue resistance, number of mitochondria, lipid content, and glycogen content (Table 1) [35]. Oxidative red fibers contain higher amounts of myoglobin for oxygen storage which is compatible with the metabolism metabolizing lipid. Conversely, glycolytic metabolism, which predominates in white fibers, contain enzymes that can readily use glycogen which are consistent with their rapid contraction speed. White fibers work in a phasic mode of action; they contract fast in short burst, so they get easily fatigued. Red fibers are slower, but last for longer time, which is referred as tonic phase [35].

Table 1.1 Characteristic of muscle fibers in domestic meat animals

	Type 1	Type 2A	Type 2X	Type 2B
Color	Red	Red	Pink	Pink/white
Contraction time	Slow	Moderately fast	Fast	Very fast
Resistance to fatigue	High	Fairly high	Medium	Low
Contractile action	Tonic	Tonic	Phasic	Phasic
Mitochondrial density	High	High	Medium	Low
Oxidative capacity	High	High	Medium	Low
Glycolytic capacity	Low	Low	Medium	High
Lipid content	High	Medium	Low	Low
Glycogen content	Low	Low	High	High
Myoglobin content	High	Medium	Low	Low

Muscle fibers are related to muscle development and meat quality [36]. Metabolic activities in normal cells rely primarily on mitochondrial oxidative phosphorylation; however, external interference in the normal activity may shift the metabolism to glycolytic, which is less efficient to produce energy [37]. Maternal nutrition may affect the amount of the fibers types, influencing the performance of the animal and production efficiency. Daniel et al. [38] showed that maternal dietary restriction during mid-gestation increases the number of fast (type II) muscle fibers, which generate energy through glycolytic pathways, as a compensatory process due to limited energy availability for muscle development.

Immune System Development

The cattle immune system begins to develop prenatally. At about 40 days of gestation, the thymus, a lymphoid organ that produces T cells for the immune system, is already evident [39]. At 70 days of gestation, it is possible to observe fetal response to some viruses [39]. By the third trimester, the calf can respond to many different infections. Few days around calving, there is an increase in the maternal and fetal steroid necessary to calving; however, it suppresses the immune responses of the calf [39]. At birth, the calf has no antibodies in the blood stream, unless infected before birth, and the immune cells functions are slower and lower than in adults. Since the immune response of the neonatal calf is still immature, it is necessary to provide adequate passive antibody transfer via colostrum. By the age of 5 to 8 months, the immune system has completely developed [39].

Few studies elucidated the immune system development prenatally in cattle and most of them focused on comparisons with other species. It has been shown that during development, cattle shared a total of 93 genes expressed by their placentae with humans. Most of those genes are related to immune system modulation (i.e. *Chemokine receptor*

activity and *negative regulation of T cell proliferation*). For example, V-set and immunoglobulin domain-containing 4 negatively regulated T-cell activation [40] is highly expressed by endometrial macrophages in the pregnant cow [41]. Another gene expressed during pregnancy is the *PD-1*, which is suggested to promote and enhance induced regulatory T-cells through antagonism of the *mTOR* cascade in naive T-cells on nonhematopoietic tissues [42].

Immune system and stress response

Stress response is associated with an interaction of systems, inducing the central nervous system, the endocrine system, and the immune system. In general, the response of the immune system is a mechanism that organisms have developed to defend against environmental challenges. The most common theory is that stress suppresses components of the immune system, thus enhancing susceptibility of an animal to disease [43].

Stress response is controlled by the hypothalamic-sympathetic and the hypothalamic-pituitary-adrenal (HPA) systems. Beginning with the hypothalamic-sympathetic system, neurons in the paraventricular nucleus of the hypothalamus cause release of catecholamines from the brain and the adrenal medulla [44]. Further, the HPA axis is activated, leading to production and secretion of corticotropin releasing factor (CRF) or corticotropin releasing hormone [44]. CRF activates endocrine cells in the anterior pituitary which start to synthesize and secrete proopiomelanocortin or its products, such as β-endorphin, adrenocorticoptropin hormone (ACTH), and melanocyte stimulating hormone. Pituitary ACTH have an effect on the adrenal cortex, where cells of the zona fasciculata secrete glucocorticoids, with cortisol being the primary glucocorticoid in cattle [45].

Glucocorticoids are produced under stress and have been shown to inhibit some components of the immune system, such as interleukin (IL) 4 (IL-4), IL-5, IL-6, IL-12, interferon gamma (IFNγ), and tumor necrosis factor-α [46,47] while increasing the secretion of other cytokines, i.e. IL-10 [47]. It seems like the inhibition of cytokines by glucocorticoids is a mechanism to avoid overshooting of the immune defenses. In general, proinflammatory cytokine synthesis are inhibited and immunosuppressive cytokines are stimulated [45]. The inhibition of IL-12 by glucocorticoids may cause a shift from a Th1 immune-driven response to a Th2 response [45]. Similar to glucocorticoids, catecholamines also inhibit IL-12 and stimulate IL-10 production [48]. Thus, glucocorticoids and catecholamines, through their effects on Th1 and Th2 cytokine secretion, may cause suppression of cellular immunity and cause a shift toward Th2-mediated humoral immunity [48].

Strategies to Modulate Pre- and Post-Natal Development

Fetal Programming and Maternal Nutrition

The early and most famous epidemiological studies in human that pointed to the possible importance of programming was the Dutch Famine birth cohort study based on the offspring of mothers exposed to famine in the Netherlands (Dutch winter famine of 1944–1945) [49]. This historical study showed strongly impaired glucose tolerance, insulin resistance, and type-2 diabetes in those exposed to the famine during gestation due to under-nutrition and uteroplacental insufficiency [49]. Therefore, there was an evidence that adverse intrauterine conditions may decrease birth weight and also impair development of the fetal endocrine pancreas, which gave rise to the “fetal origins of adult disease” based on the fetal programming phenomenon [50].

Fetal programming, also called developmental programming, is the response to a specific alteration to the maternal organism during pregnancy which can alter fetal physiological functions during fetal development, and have lasting consequences in the adult life [51].

Fetal nutrient deficiency has been associated with impaired fetal growth and predisposition to chronic disease such as coronary heart disease, stroke, diabetes and hypertension [52]. Similarly, fetal programming in livestock can cause a lack of optimal fetal growth affecting production efficiency [21].

Maternal nutrient deficiency has been noted as one of the main factors affecting fetal growth and development [53]. For example, cows subjected to nutrient deficiency during pregnancy gave birth to fatter offspring compared with control animals [54–56]. Another study, in which ewes received 50% (nutrient restricted) or 100% (control fed) of total digestible nutrients during mid gestation, the restricted group showed reduced muscle development and fewer secondary myofibers [57], showing long-term effects on offspring performance. Maternal nutrition also affected the intramuscular and visceral fat increasing the contents in skeletal muscle of nutrient restricted lambs [38,57]. Similar to undernutrition, overnutrition can also affect fetal development. Steers born from cows fed high-protein pasture in mid to late gestation had heavier body weights and hot carcass weights than control animals. Also, there was an increase in meat tenderness and 12^{th} rib fat thickness in calves born from cows fed high-protein diets [58]. These results show that improving nutritional status of cows during mid to late gestation positively affects tenderness, adipose tissue deposition, and growth in steers [58].

Studies have shown that the uterine environment may impact the regulation of genes in the offspring, and thus, affect the homeostatic regulatory mechanisms in the adult life. Jennings et al. [59] showed that altered energy supply during mid-gestation affects genes regulating fetal adipose and muscle development in cattle. For example, the *Longissimus dorsi* muscle of fetuses, collected from cows receiving 146% of the energy requirements from 85 to 180 days of gestation, had higher expression ($P < 0.05$) of *Pref-1* compared with to those collected from cows receiving 87% of the energy requirement. The expression of *C/EBP-β* was higher ($P < 0.05$) in fetus of cows receiving low energy (72%) compared with the group receiving 87%. Similarly, Lan et al. [60] showed significant effects of the different maternal diets (alfalfa haylage versus corn) on the expression of genes in fetal tissue of sheep. The expression of *IGF2R* and *H19* were higher in fetuses born from dams receiving alfalfa haylage compared to those born from dams receiving corn, probably due to the low amino acid content in the corn diet. These results provide evidences of association between maternal nutrition during pregnancy and the transcriptomic profile of fetal tissues.

Maternal nutrition during pregnancy plays an essential role in the proper development of the placenta and the fetus. Therefore, a good understanding of how maternal diet can improve or delay this development is essential for increased production efficiency. Studying the response of genes to maternal over-feeding and nutrient restriction during mid-gestation could demonstrate how management decisions influence fetal adipose and muscle development.

Preconditioning Phase

Preconditioning phase is the period immediately after weaning, in which the animals experience stressful events such as feedlot entry and vaccination, including Infectious Bovine Rhinotracheitis, Parainfluenza3, Bovine Viral Diarrhoea, and Bovine Respiratory Syncytial Virus vaccines [3]. The stress process promotes the production of proinflammatory cytokines, predominately IL-1, IL-6, and tumor necrosis factor in the animal, stimulating the hepatocytes to produce acute-phase proteins [12]. This activated immune response results in an increased mobilization of protein from muscle [13] to increase the synthesis of proteins and cells involved in the immune system [14]. Consequently, growth performance is compromised due to limited availability of nutrients to skeletal muscle development. Also during this period, beef calves may experience decreased energy and protein intake [3] which may reduce the immune capacity of the animal and, consequently, impacting their vaccination response [12].

Management strategies have been shown to reduce the impact of the acute-phase response and benefit dry matter intake, body weight gain, and production efficiency parameters in beef cattle [61]. Cooke et al. [11] showed that inclusion of a rumen-protected poly-unsaturated fatty acid (PUFA) source in preconditioning diets reduced the TNF-α response triggered by transport and feed yard entry, and benefited feedlot performance and carcass marbling of feeder steers. Therefore, PUFA supplementation during preconditioning might be a feasible alternative to enhance immunological and performance variables in feeder cattle. Also, early-weaning calves at 70 to 90 days of age has been reported to improve feedlot performance through reduction of plasma concentrations of acute phase proteins [62].

There is no doubt that the stimulation of the stress process and the immune response during acute phase response stimulates cytokines which disrupt the normal regulation of

metabolism in general, and protein turnover and amino acid metabolism [14]. However, management strategies have been shown to reduce the impact of the acute-phase response and benefit the performance of the animal [61].

Gene Expression

Gene Expression Regulation

Gene expression is regulated by fundamental cellular processes such as transcription, mRNA degradation, and translation. Each of these steps is controlled by gene-regulatory events. The first step of gene expression is the DNA transcription in which DNA is copied into RNA by the enzyme RNA polymerase. The regulation of gene expression is also related to the degradation of mRNA [63]. Both of these processes have been shown to be regulated by RNA interference or RNA silencing, which promote or impairs translation by silencing cytoplasmic mRNAs, either by triggering an endonuclease cleavage, or accelerating mRNA decapping [64]. Also, epigenetic modifications can control gene expression through cytosine methylation and histone modifications [65]. RNA translation corresponds to the synthesis of proteins directed by a mRNA template. The RNA sequence is read in three nucleotides "frame", called codons, which encode for one amino acid. Translational regulation of mRNA is an important step in the control of gene expression [66]. This regulation can be controlled by the available amount of ribosomes or initiation factors [67] or, more often, by a change in the activity of these factors through phosphorylation or dephosphorylation [67].

Epigenetic Modification

All process in the organism, including myogenesis, adipogenesis, and fibrogenesis, are controlled by the expression of one or more crucial genes. Enhancing or silencing expression of genes is essential for maintaining the diversity of cells and cellular functions. This control may be accomplished through epigenetic modifications [68].

Epigenetic modifications refer to cytosine methylation and histone modifications and they are heritable [65]. Histone modification can cause stable alterations in gene expression [69] although it only passes for few generations [70]. Polycomb group proteins (PcG) and trithorax (trxG) group proteins regulate histone methylation, specifically of histone H3, leading to epigenetic modifications during cell differentiation [71]. PcG group proteins possess H3K27-specific trimethylase activity which mediates gene expression repression, whereas trxG complexes have H3K4 trimethylase activity which mediates activation of genes [68]. Several mechanisms can lead to gene silencing through DNA methylation: 1) recruitment of histone deacetylases, which removes histone acetylation, which increases the affinity between histones and DNA, inhibiting gene expression; 2) DNA methylation can directly interfere with the binding of transcription factors; and 3) DNA methylation leads to the formation of inactive chromatin structure [71].

Currently, indirect line of evidences supports epigenetic modification in regulating key genes controlling fetal muscle development (Figure 1.3). For example, maternal diet alters the expression of PPARs in fetal muscle through DNA methylation [72]. In another study, maternal cocaine administration causes epigenetic modification of a key protein kinase gene in rat heart [73]. In addition to the analysis of the transcriptome, Lan et al. [60] also studied the differential expression of imprinted genes and DNA methyltransferase genes as an effect of maternal diets on the expression of these genes. The methylation levels of CpG

islands of both *IGF2R* and H19 were higher in fetuses born from dams receiving alfalfa haylage compared with those born from dams receiving corn. Likewise, Cooney et al. [74] showed that maternal methyl supplements in mice increased the level of DNA methylation in the *agouti* LTR with positive effects to the health of the offspring. These results provide evidence of association between maternal nutrition during pregnancy and epigenomic alterations in the fetal tissues and, consequently in the adult life.

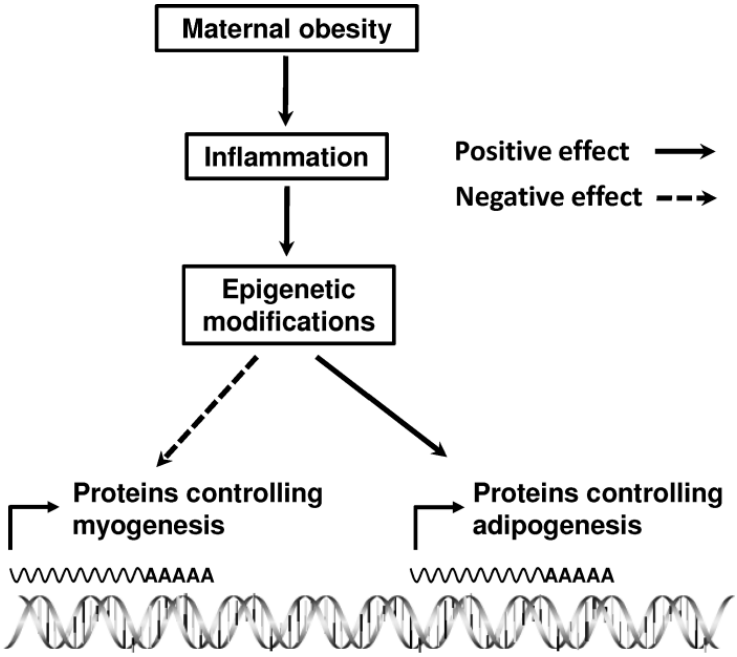

Figure 1.3 *Epigenetic modification as an effect of maternal nutrition. Maternal obesity may induce epigenetic modifications that alter the expression of genes involved in myogenesis and adipogenesis in mesenchymal stem cells* [21].

RNA-seq

Introduction to RNA-seq

Several technologies have been used for transcriptome profiling, including hybridization methods, such as DNA microarrays [75,76], and sequencing-based approaches like SAGE (Serial Analysis of Gene Expression) [77] and MPSS (Massively Parallel Signature Sequencing) [78].

DNA microarrays are based on the hybridization of mRNA extracted from biological samples to a pre-selected mRNA library, which can contain up to tens of thousands of various mRNA transcripts [79]. Then, the intensities of hybridization signals are read to obtain the expression level of each transcript. Some advantages of hybridization-based approaches include a significantly lower workload and relatively low cost [79]. However, these methods have several limitations including necessity of existing knowledge about genome sequence, high background levels owing to cross-hybridization, and a limited dynamic range of detection owing to both background and saturation of signals [80].

Sequencing-based methods do not require any pre-compilation of an mRNA library of sequences, but instead, they use restriction enzymes type, i.e. tagging enzymes, to collect short tags (typically 10–22 bases) from each mRNA molecule, provided a relevant recognition site exists for an anchoring enzyme. Then, either by sequencing long tags using conventional sequencer (SAGE), or by performing iterative parallel sequencing using a proprietary technique (MPSS), it is possible to determine the identity of a sufficiently large amount of tags in an efficient manner. The abundance of each mRNA transcript is assumed to be proportional to the count of occurrence of its representative tag. As an advantage, these technologies allow the identification of novel mRNAs; however, they require more advanced instruments that are higher cost, and more labor-intensive, only a portion of the transcript is analyzed, and isoforms are generally indistinguishable from each other [79]. These disadvantages limit the use of traditional sequencing technology in annotating the structure of transcriptomes.

Recently, the development of novel high-throughput RNA sequencing method (RNA-seq) has provided a new technique for mapping and quantifying transcriptomes. This new

technology offers several advantages over existing methods. First, comparing with hybridization-based approaches, RNA-seq is not limited to detecting transcripts that correspond to existing genomic sequence; it can reveal the precise location of transcription boundaries and also reveal sequence variations (for example, single nucleotide polymorphisms - SNPs) in the transcribed regions [81,82]. Relative to DNA microarrays, RNA-seq has very low, if any, background signal because DNA sequences can be unambiguously mapped to unique regions of the genome and the quantification correlates with the number of sequences obtained. Consequently, it has a large dynamic range of expression levels over which transcripts can be detected: a greater than 9,000-fold range was estimated in a study that analyzed 16 million mapped reads in *Saccharomyces cerevisiae* [83] and a range spanning five orders of magnitude was estimated for 40 million mouse sequence reads [84]. In addition, RNA-seq has also been shown to be highly accurate for quantifying expression levels, as determined using quantitative PCR (qPCR) [83]. It also shows high levels of reproducibility, for both technical and biological replicates [81,83].

Considering these advantages, RNA-seq is the first sequencing-based method that allows the entire transcriptome to be quantified with much lower cost than either tiling arrays or large-scale Sanger expressed sequencing tag. On the other hand, like other high-throughput sequencing technologies, RNA-seq also faces several informatics challenges, including the development of efficient methods to store, retrieve and process large amounts of data [85]. The advance of technology and the development of new sequencing platforms are decreasing the costs of sequencing, allowing RNA-seq to replace microarrays for many applications that involve determining the structure and dynamics of the transcriptome.

Processing RNA-seq Data

RNA-seq uses a recently developed deep-sequencing technologies. In general, a population of RNA (total or fragmented) is converted to a library of cDNA fragments. Then, adaptors are attached to one or both ends for amplification. [85]. Next, each molecule, with or without amplification, is sequenced in a high-throughput manner to obtain short sequences from single-end (SE) sequencing or both ends (paired-end; PE) sequencing [85]. The reads range between 30–400 base pairs (bp), depending on the DNA-sequencing technology used. Depending on the genome availability for the organism being studied and the research goal, the reads obtained from the sequencing process are either aligned to a reference genome or reference transcripts, or assembled *de novo*.

For large transcriptomes, alignment is complicated because a significant portion of sequence reads matches multiple locations in the genome. In case of low-copy repetitive sequences, it is possible to overcome this issue assigning these multi-matched reads by proportionally assigning them based on the number of reads mapped to their neighboring unique sequences [81,84]. However, short reads with high copy numbers and long stretches of repetitive regions represent a greater challenge. One strategy would be to employ paired-end sequencing, in which short sequences are determined from both ends of a DNA fragment [86–88] and extend the mapped fragment length to 200–500 bp.

There are many RNA-seq protocols and analyses available and there is no optimal pipeline for all experiments. Every RNA-seq experimental scenario could potentially have different optimal methods for transcript quantification, normalization, and differential expression analysis [89].

To start with, it is important that the experimental design for the RNA-extraction protocol being used remove the highly abundant ribosomal RNA (rRNA), which typically

constitutes over 90% of total RNA in the cell, leaving the 1–2% comprising messenger RNA (mRNA) that we are normally interested in [90]. For this, there are two options: 1) to enrich for mRNA using poly(A) selection or 2) to deplete rRNA.

Furthermore, sequencing can involve SE or PE reads. The best sequencing option depends on the analysis goals. PE reads are preferable for *de novo* transcript discovery or isoform expression analysis [91,92]. The cheaper, short SE reads are normally sufficient for studies of gene expression levels in well-annotated organisms [90].

Finally, a crucial design factor is the number of replicates. The number of replicates that should be included in a RNA-seq experiment depends on several factors, for example, the amount of technical variability in the RNA-seq procedures, the biological variability of the system under study, and the desired statistical power [90].

Quality control and trimming

Before starting the alignment process of raw reads in the genome, it is necessary to analyze the quality of the reads that were generated from sequencing. This quality control involves the analysis of sequence quality, GC content, the presence of adaptors, overrepresented k-mers and duplicated reads in order to detect sequencing errors, PCR artifacts or contaminats [90].

Acceptable duplication, k-mer, and GC content levels depend on the experiment and the organism being studied, but these values should be homogeneous for samples in the same experiments. Also, it is recommended that outliers with over 30% disagreement be discarded. In general, read quality decreases towards the 3’ end of reads, and if it becomes too low, bases should be removed to improve alignment [90].

FastQC [93] is a popular tool to perform quality control on Illumina reads, whereas NGSQC [94] can be applied to any platform. FastQC imports data from BAM, SAM or FastQ files and provides a quick overview to tell you in which areas there may be problems. The results are summarized in graphs and tables to quickly assess your data and can be exported to an HTML based permanent report.

In case of low quality, software tools such as the FASTX-Toolkit [FASTX-Toolkit. http://hannonlab.cshl.edu/fastx_toolkit/] and Trimmomatic [95] can be used to discard low-quality reads, trim adaptor sequences, and eliminate poor-quality bases. In addition, the online website PrinSeq (https://sourceforge.net/projects/prinseq/) allows us to filter, reformat, or trim genomic and metagenomic sequence data. It generates summary statistics of sequences in graphical and tabular format, providing a user-friendly interface. Sequence data can be filtered to remove sequence copies, sequences with N's, and low-quality sequences.

Identification of transcripts

When a reference genome is available, RNA-seq analysis will normally involve the mapping of the reads onto the reference genome or transcriptome to infer which transcripts are expressed. Mapping the reads to known genome or transcriptome focuses the analysis on quantification only. On the other hand, if the organism does not have a sequenced genome, then the analysis is based on assembling reads into longer contigs and treating these contigs as the expressed transcriptome. Then, the reads are mapped again to the de novo transcriptome for quantification. In either case, read coverage can be used to quantify transcript expression level [90].

Reads may be assigned to only one position in the reference or multi-mapped (i.e multireads). Multireads occur due to repetitive sequences or shared domains of paralogous

genes. They normally account for a significant fraction of the mapping output and should not be discarded. It is even more common if the reads are being mapped to the transcriptome because a read that would have been uniquely mapped on the genome would map equally well to all gene isoforms in the transcriptome that share the exon. In both cases, genome and transcriptome mapping, transcript identification and quantification become important challenges for alternatively spliced genes [90].

Mapping to a reference genome allows for the identification of novel genes or transcripts and requires the use of a gapped or spliced read mapper as reads may span splice junctions. The challenge is to identify splice junctions correctly, especially when sequencing errors occur, differences within the reference exist or when non-canonical junctions and fusion transcripts are sought. TopHat is one of the most popular RNA-seq mappers and follows a two-step strategy: 1) unspliced reads are first mapped to exons, and then 2) unmapped reads are split and aligned independently to identify exon junctions [96,97].

Many mappers exist that are optimized for different functions. For example, there are mappers specialized in identifying SNPs (i.e. GSNAP [98], PALMapper [99], and MapSplice [85]), detecting non-canonical splice junctions (i.e. STAR [100] and MapSplice [85]), and achieving ultra-fast mapping (i.e. GEM [101], and to map long-reads, i.e. STAR [100]). All of these aligners are called splice-aware aligners since they would not to try to align RNA-seq reads to introns and identify possible downstream exons and try to align to those instead, ignoring introns altogether. However, if the transcriptome annotation is comprehensive, it is possible to choose to map directly to a FASTA-format file of all transcript sequences for all genes of interests. In this case, no gapped alignment is needed and unspliced mappers such as Bowtie [96] can be used.

It is important to consider some parameters during alignment, such as the number of mismatches to accept, the length and type of reads (SE or PE), and the length of sequenced fragments during mapping.

Quantification

The most common application of RNA-seq is to estimate gene and transcript expression. This application is primarily based on the number of reads that map to each transcript sequences. The simplest approach to quantification is to aggregate raw counts of mapped reads using programs such as HTSeq-count [102] or featureCounts [103]. This gene-level (rather than transcript-level) quantification approach utilizes a gene transfer format (GTF) file containing the genome coordinates of exons and genes, and often discards multireads. Raw read counts alone are not sufficient to compare expression level among samples, as these values are affected by factors such as transcript length and total number of reads sequenced. [90].

Correcting for gene length is not necessary when comparing changes in gene expression within the same gene across samples, but it is necessary for correctly ranking gene expression levels within the sample to account for the fact that longer genes accumulate more reads. Furthermore, programs such as Cufflinks that estimate gene length from the data can find significant differences in gene length between samples that cannot be ignored [90].

Statistical Analysis of RNA-seq Data

Normalization of the Data

Differential expression analysis requires that gene expression values be compared among samples [90]. The normalization of the data is important to account for the sequencing depth and the number of transcripts, which can differ significantly between samples [104]. Also, the number of fragments expected to map to a gene depends on the composition of the RNA population. For example, if many reads are being mapped to only one gene or one gene is highly expressed in one experimental condition, the remaining genes will be underestimated; therefore, we need to account for this difference [90]. Most normalization methods use the raw data to estimate appropriate scaling factors that can be used in downstream statistical analysis procedures, thus accounting for the sampling properties of RNA-seq data [89].

Many normalization methods have been described to deal with RNA-seq data. The differences among them is regarding the type of bias adjustment and the statistical strategy adopted. Some examples of normalization methods are: Trimmed Mean of M values (TMM) [105], DESeq [106], Upper Quartile (UQ) [107], Median (Med) [107], and Reads Per Kilobase per Million mapped reads (RPKM) normalization [84]. The normalization methods can be divided into two groups based on the library size concept (TMM and DESeq), or on the distribution adjustment of read counts (UQ, Med, RPKM).

The TMM normalization method [105] is based on the hypothesis that most genes are not differentially expressed. This method is implemented in the *TCC* Bioconductor package (version 1. 0. 143) [108]. Normalization factors are calculated by selecting one sample as a reference and calculating the TMM factor for each non-reference sample [89]. In this method, it is not possible to directly estimate the total RNA production since the expression

levels and true lengths of every gene are unknown. However, the relative RNA production of two samples, *i* and *j*, can be determined by:

$$f_k = \frac{S_{ki}}{S_{kj}}$$

where f_k is the relative RNA production in library k and S_k is the total RNA production. The TMM method uses a weighted trimmed mean of the log expression ratios to the ratio of RNA production. For example, for sequencing data, the gene-wise log fold changes (M value) is defined:

$$M_g = log_2 \frac{Y_{gki}/N_{ki}}{Y_{gkj}/N_{kj}}$$

where M_g is the M value of gene g; Y_{gk} is the observed count for gene g; and N_k is the total number of reads for library k. The absolute expression levels (A value) is defined:

$$A_g = {}^1\!/_2 \, log_2 \left(\frac{Y_{gki}}{N_{ki}} * \frac{Y_{gkj}}{N_{kj}} \right)$$

Before taking the weighted average, both values are trimmed. Precision (inverse of the variance) weights are used to account for the fact that log fold changes from genes with larger read counts have lower variance on the logarithm scale. Normalization factors across several samples can be calculated by selecting one sample as a reference and calculating the TMM factor for each non-reference sample. The TMM normalization factors can be built into the statistical model used to test for DEG. For example, a Poisson or negative binomial model would utilize an effective library size to adjust the modeled mean. It can be done using an additional offset in a generalized linear model.

The DESeq normalization method [102] is also based on the hypothesis that most genes are not differentially expressed and is included in the *DESeq* Bioconductor package

(version 1.6.0) [102]. A *DESeq* scaling factor for a given lane is computed as the median of the ratio for each gene of its read count over its geometric mean across all lanes. The underlying idea is that non-DEG should have similar read counts across samples, leading to a ratio of 1. Assuming that most genes are not differentially expressed, the median of this ratio for the lane provides an estimate of the correction factor that should be applied to all read counts of this lane to fulfill the hypothesis. In the UQ normalization method, the gene counts are divided by the upper quantile of counts associated with their lane and multiplied by the mean total count across all the samples of the dataset [106]. The Med method is similar to UQ, but the counts are divided by the median counts different from 0 in the computation of the normalization factors. Lastly, the RPKM combines between- and within-sample normalization, as it re-scales gene counts to correct for differences in both library sizes and gene length [84]. However, it has been shown that this method may introduce bias in the per-gene variances, especially for lowly expressed genes [109]. Normalization is an essential step in the analysis of gene expression [105,106] and many methods are available for this purpose.

Differential Expression

High-throughput sequencing of RNA fragments has been used with the purpose of quantifying gene expression. In general, reads obtained from sequencing are mapped to a target genome classified in target transcript, in the case of RNA-seq. The read count has been found to be – to good approximation – linearly related to the abundance of the target transcripts [84], which represents an important summary statistic of the number of reads aligned to a gene or transcript.

In general, RNA-seq analysis involves a very large number of genes to be tested, a small number of biological samples due to costs restrictions, and technical and biological variances which makes it a challenge to model the data [102]. One of the main interest of sequencing is to compare read counts between different biological conditions. To achieve this goal, statistical tests are used to test if an observed difference in read counts is significant, that is, whether it is greater than what would be expected just due to natural random variation. Among the statistical models used for this purpose, models assuming Poisson and negative binomial distributions have been widely used [85,110].

The Poisson distribution has the advantage of being simple, since it has only one parameter, where the variance of the model is equal to the mean [102]. Therefore, it can only account for the technical noise in RNA-seq data, predicting small variances (Figure 1.4) [111].

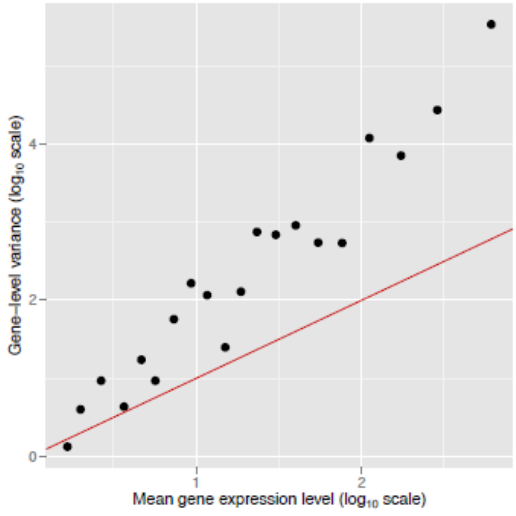

Figure 1.4 *Relationship between mean and variance of RNA-seq data using Poisson regression. Each point represents simulated data for one biological replicate. The red line is the variance implied by the Poisson distribution* [112].

The assumption of genes having the same mean and variance is too restrictive. In RNA-seq data, the variance is generally greater than the mean, a phenomenon called overdispersion which result in a lack of control of type-I error (the probability of false discoveries). To address this issue, it has been proposed the model count data with negative binomial (NB) distributions [113] as an alternative which includes an additional dispersion parameter.

Negative Binomial Model

Negative binomial models have been used as an alternative approach when overdispersion occurs with Poisson generalized linear models [114]. The NB distribution has two parameters, encoding the mean and the dispersion, which allows the modeling of more general mean–variance relationships [102]. Anders and Huber [102] plotted the variance against the mean for the NB and Poisson using RNA-seq data, and showed that the NB model presented a better fit (Figure 1.5).

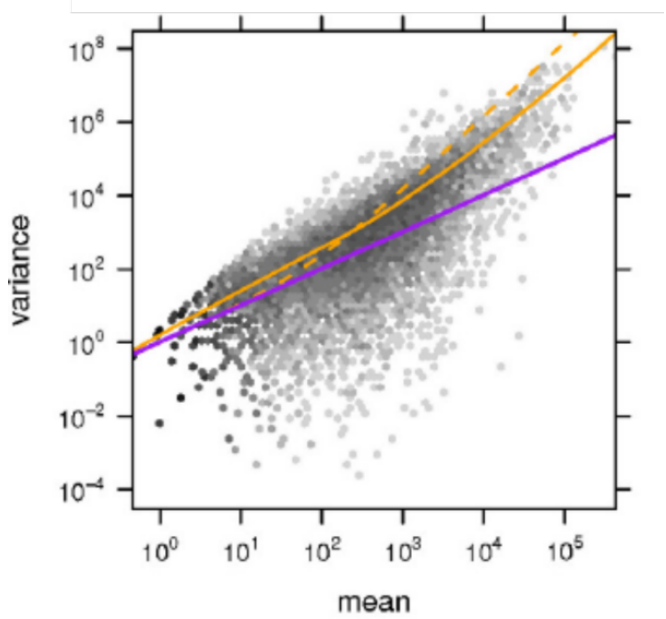

Figure 1.5 *Comparison of the relationship between mean and variance of RNA-seq data using Poisson vs NB regression. Dependence of the variance on the mean for one condition in RNA-seq data. The scatter plot shows the transformed values to a common-scale sample count data of the variance plotted against the transformed values of the mean. The orange line is the fit variance-mean dependence by DeSeq (NB approach). The purple lines show the variance implied by the Poisson distribution for each of the two samples. The dashed orange line is the variance estimate used by edgeR (NB approach)* [102].

Using a mix of a Poisson process with a gamma distribution for the Poisson parameter results in a distribution which is over dispersed relative to the Poisson [114]. For a given Poisson distribution *Y* with mean λ, λ follows a gamma distribution, $G(k, \mu)$ with probability density function [114]:

$$f(\lambda; \mathrm{k}, \mu) = \frac{(k/\mu)^k}{\Gamma(k)} \exp\left(-\frac{\mathrm{k}\lambda}{\mu}\right) \lambda^{k-1}, \qquad \lambda \geq 0$$

and $E(\lambda) = \mu$ and $Var(\lambda) = \frac{\mu^2}{k}$, where *k* describes the shape of the distribution. The density is skewed to the right and the degree of skewness decreases as *k* increases [114].

Marginally, the gamma mixture of the Poisson generates the NB distribution for *Y* [115], with probability mass function:

$$p(y; k, \mu) = \frac{\Gamma(y+k)}{\Gamma(k)\Gamma(y+1)} \left(\frac{k}{\mu+k}\right)^k \left(1 - \frac{k}{\mu+k}\right)^y, \quad y = 0, 1, 2, \ldots$$

The dispersion parameter $\gamma = 1/k$, and $E(Y) = \mu$ and $Var(Y) = \mu + \gamma\mu^2$. As γ goes to 0, the variance approximates to the mean, and it becomes a Poisson distribution [114].

The NB model for count data allows μ to depend on explanatory variables. On the other hand, γ is usually equal for all observations and correspond to the coefficient of variation in the gamma distribution [114]:

$$\frac{\sqrt{var(\lambda)}}{E(\lambda)} = \sqrt{\gamma}$$

False Discovery Rate (FDR)

The FDR procedure was described by Yoav Benjamini and Yosef Hochberg in 1995 with the purpose of controlling type-I error (probability of false rejecting null hypotheses) in multiple comparison procedures [116]. By definition, the *P*-value is the probability of finding a result equal to or more extreme than what was actually observed when the null hypothesis of a study question is true. Similarly, in multiple testing, we can use the adjusted *P*-value (*q*-value) to define the level in which the multiple testing procedure would reject the hypothesis [117]. Therefore, the multiple testing problem can be overcome by adjusting the *P*-values (Figure 1.6).

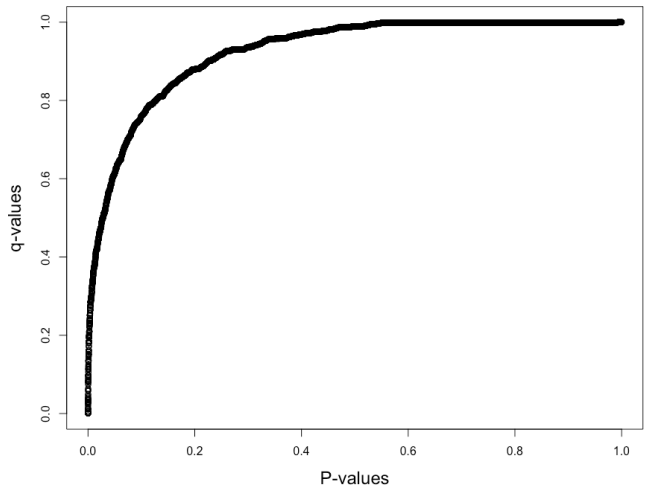

Figure 1.6 *Relationship between raw and adjusted p-values (q-value). Each dot represents the test for the effect of diet (restricted versus control) in the muscle tissue described in Chapter 2.*

To calculate adjusted *P*-values, it is necessary to sort *P*-values in ascending order, such as $P\text{-value}_1$, $P\text{-value}_2$, …, $P\text{-value}_m$, considering m multiple tests, where the 1st *P*-value is the smallest and the m^{th} the largest. Then, each *P*-value is multiplied by an adjustment factor, given by $a_i = m/i$ being i the position of the *P*-value (*P*). Then, the *q*-value [117] is given by:

$$q - value = a_i * P_i$$

In case the multiplication in this step results in a smaller q-value than what was obtained for the previous P-value, the previous q-value will be substituted by the smallest q-value [117].

Covariance Structures

Comparative experiments with repeated measures consist of samples collected in a longitudinal study in which measurements from each subject are made at multiple time points or under different conditions. Measures on the same subject at different times (or different conditions) may be correlated, with measures taken close together in time being more highly correlated than measures taken far apart in time. Therefore, it is important to account for the covariance structure of the data, which usually reflects a series of measurements collected over time. Different subjects are often assumed independent, although this assumption depends on the study design. Several covariance approaches are available in the literature [118].

Considering Y_{ij} the response on subject j in group i, the simple (SIM) or uncorrelated structure specifies that observations (Y) are independent, even on the same subject, satisfying:

$$Var(Y_{ijk}) = \sigma^2_{SIM}$$

$$cov(Y_{ijk_1}, Y_{ijk_2}) = 0$$

where k corresponds to different times in which a measurement was taken on the same subject. In this covariance structure, homogeneous variance is assumed for all observations, such that $Var(Y_{ijk}) = \sigma^2_{SIM}$ [118].

A traditional covariance structure used in repeated records is the compound symmetric (CS), which specifies that observations on the same individual have homogeneous variance and covariance, such that:

$$Var(Y_{ijk}) = \sigma^2_{CS,b} + \sigma^2_{CS,w}$$

$$cov(Y_{ijk_1}, Y_{ijk_2}) = \sigma^2_{CS,b}$$

where $\sigma^2_{CS,b}$ and $\sigma^2_{CS,w}$ are the between-subjects and within subject variances, respectively. In the case of repeated measures across time using CS, the correlation between measurements does not depend on the length of the time interval between measurements, and is equal to the between-subjects variance [118]. Assuming three measurements on the same subject, the CS (co)variance matrix can be written as:

$$\begin{bmatrix} \sigma^2_{CS,b} + \sigma^2_{CS,w} & \sigma^2_{CS,b} & \sigma^2_{CS,b} \\ \sigma^2_{CS,b} & \sigma^2_{CS,b} + \sigma^2_{CS,w} & \sigma^2_{CS,b} \\ \sigma^2_{CS,b} & \sigma^2_{CS,b} & \sigma^2_{CS,b} + \sigma^2_{CS,w} \end{bmatrix}$$

The first-order autoregressive (AR[1]) covariance structure specifies a homogeneous variance for all measures, whereas the covariance between two observations on the same subject depends on the length of the time interval between measurements, in way that it decreases as measures are taken further apart, such as:

$$Var(Y_{ijk}) = \sigma^2_{AR(1)}$$

$$cov(Y_{ijk_1}, Y_{ijk_2}) = \sigma^2_{AR(1)} \rho^{|l-m|}_{AR(1)}$$

where ρ represents the correlation between the measurements k_1 and k_2 [118] and l and m are the (m, l)th element of the matrix. Assuming three measures taken over time, the (co)variance matrix can be represented by:

$$\sigma^2_{AR(1)}\begin{bmatrix} 1 & \rho^{|l-m|}_{AR(1)} & \rho^{|l-m|}_{AR(1)} \\ \rho^{|l-m|}_{AR(1)} & 1 & \rho^{|l-m|}_{AR(1)} \\ \rho^{|l-m|}_{AR(1)} & \rho^{|l-m|}_{AR(1)} & 1 \end{bmatrix}$$

Functional Analysis

Functional analyses are primarily designed to handle a gene list data from any genome-wide experiments and associate the genes to annotated biological functions or pathways. Statistical overrepresentation tests are based the simple binomial model [119], in which a test gene list uploaded by the user (i.e. DEG from RNA-seq experiments, or list of genes with significant *P*-values from genome-wide association experiment), is compared to a reference gene list to determines whether a particular class (i.e. a Gene Ontology (GO) biological process) of genes is overrepresented or underrepresented [120].

The input (or test) list correspond to the list of genes and it is divided into groups based on GO classification i.e. molecular function, biological process, and KEGG pathway. Then, the list is compared to a reference list respective to the species of interest which is also divided into groups in the same way. Next, a binomial test is applied for each functional category (for example, protein kinase for molecular function, cell proliferation for biological process or apoptosis signaling pathway for pathway) to determine whether there is a statistical overrepresentation or underrepresentation of the genes in the test list relative to the reference list. For that, the reference genome has a number of expected genes associated with each category. Based on this number, it is possible to calculate the number of genes that

would be expected to be present in the test list for each category. This expected number is compared with the number of genes observed in the list of genes provided. When there are more observed than expected genes, there is an overrepresentation of genes involved in the specific function under investigation. If fewer genes are observed than expected, there is an underrepresentation [120].

It is necessary to test if the over- or underrepresentation is occurring by random chance, which can be evaluated based on a *P*-value from a binomial test. The 'null' hypothesis assumes that genes in the test list are sampled from the same general population as genes from the reference set, and thus the probability *P(C)* of observing a gene from a particular category *C* in the test list is the same as in the reference list [120]. First, for the reference list, the probability *P(C)* is

$$P(C) = \frac{n(C)}{N}$$

where *n(C)* is the number of genes mapped to category *C*, and *N* is the total number of genes in the reference set. Under the null hypothesis, the number of genes mapped to *C* in the test list, *k(C)*, is distributed binomially with probability parameter *P(C)*, and thus the P-value would be

$$P - value = \sum \frac{K}{k} P(C)^k (1 - P(C))^{K-k}$$

where *K* is the total number of genes in the test list and k is the number of genes in a specific category. Finally, a *P*-value cutoff is used to determine overrepresentation, i.e. when the number of observed genes *k(C)* is greater than expected *P(C)*, or underrepresentation, i.e. when *k(C)* is smaller than *P(C)* [120].

Construction of Genes Networks

A network can be used for visualization of statistical information such as correlation matrix in which each variable, i.e. gene, is a node and each correlation an edge [121]. Thus, a gene-gene network constructed by correlation between the expression levels of genes may include hundreds to thousands of correlation estimates. The most common type of correlation is the Pearson's correlation coefficient, which compares two ratio variables. The Pearson's correlation coefficient (ρ) between two random variables (i and j), can be defined as [122]:

$$\rho_{ij} = \frac{cov(i,j)}{\sigma_i * \sigma_j} \qquad \text{where} \qquad -1 \leq \rho_{ij} \leq 1$$

This correlation coefficient reveals the strength of the linear relationship between the two random variables. So, if $\rho_{ij} = 0$, then i and j are said to be uncorrelated. The closer the value of $|\rho|$ is to 1, the stronger is the correlation between the two variables [122].

Biologically, the expression of a given gene may be a function of the activity of several other genes. In this case, the correlation between the expression levels of a pair of genes must be accounted for by the expression levels of other genes. In this case, we may use partial correlation coefficients to construct gene networks. The partial correlation (ω) between two random variables (i and j) given another random variable, namely z ($w_{ij,z}$), can be obtained by [121]:

$$\omega_{ij,z} = \frac{\rho_{ij} - \rho_{iz}\rho_{jz}}{\sqrt{(1 - \rho_{iz}^2)(1 - \rho_{jz}^2)}}$$

Partial correlation indicates correlation between two variables disregarding the effects of another variable, or several other variables, on this relationship [121]. For example, in an experiment with two treatments and in which the goal is to construct a partial correlation for the 10 most DEG, the partial correlation matrix may be constructed based on the difference of expression between the two treatments.

Partial correlation networks are being used to represent gene co-expression networks. In these gene networks, each gene is represented as a node and is connected to each other with a line called an edge. The strength of the correlation can be represented by the thickness of the edge. The gene networks help to identify co-expressed (or connected) genes which can be used to explain patterns seen across experimental conditions. For example, Kogelman et. al [123] applied a weighted gene co-expression network analysis to detect clusters of highly co-expressed genes involved in transcriptional regulations of obesity from RNA-seq data in pig adipose tissue.

Final Remarks

Alterations in the maternal nutrition during pregnancy affect the intra-uterine environment, delaying or enhancing fetal development. Specially in late gestation, when there is an increase in the nutritional requirements by beef cows, a restriction in the maternal diet cause a lack of nutrients for optimum fetal development. This nutrient deficiency during the fetus development may affect the regulation of genes associated with muscle development and immune system in the progeny, affecting its post-natal performance. Therefore, we hypothesize that maternal energy restriction would cause changes in the expression of genes related to muscle development and immune response in the muscle and blood of beef calves born from cows with contrasting levels of dietary energy the last 40 days

of gestation. In the following chapter, we will describe a study in which we have used RNA-seq data to identify differentially expressed genes between the control (100% of the energy requirements) and restricted (70% of the energy requirement) groups. The biological functions and relationships of the differentially expressed genes identified from this analysis were further explored using enrichment analysis and gene-gene networks were constructed.

CHAPTER 2. IMPACT OF ENERGY RESTRICTION DURING LATE GESTATION ON MUSCLE AND BLOOD TRANSCRIPTOME OF BEEF CALVES

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Author's Contributions

LPS: performed data analyses, interpreted results, and drafted manuscript; MN: assisted with data analysis; PM: designed and carried out animal trial, and collection of samples; JS: directed RNA extraction and sequencing; MA: directed RNA extractions and sample collection; MHP: designed the animal trial; MSD: developed RNA-seq study and interpreted results; NVLS: developed RNA-seq study, data analyses, interpretation of results, and drafted manuscript. All authors read and approved the final version of the manuscript.

Abstract

Background

Maternal nutrition has been identified as one of the main factors affecting intra-uterine environment. Nutritional deficiency may impact the regulation of genes associated with myogenesis and immune response in the fetus. The increase in nutritional requirements by beef cows during late gestation can cause a lack of nutrients for optimum fetal development. Forty days before the expected calving date, cows were randomly assigned to one of two isonitrogenous diets: 100% (control group) or 70% (restricted group) of the daily net energy requirement. Muscle samples were collected at 21 days post-weaning (dpw) from 12 heifers and 12 steers, and blood samples were collected at 0, 3, 6, and 15 dpw (12 steers). The objective of this work was to identify and assess the biological relevance of differentially expressed genes (DEG) in skeletal muscle and blood of beef calves born from cows that experienced 30% energy restriction during the last 40 days of gestation compare to unrestricted controls.

Results

A total of 160, 164, and 346 DEG (q-value < 0.05) were identified in the skeletal muscle for the effects of diet, sex, and diet-by-sex interaction, respectively. For blood, 452, 1392, and 155 DEG were identified for the effects of diet, time, and diet-by-time interaction, respectively. For skeletal muscle, results based on diet identified genes involved in the muscle metabolism. In muscle, the 10 most down-regulated DEG in the energy-restricted group (REST), included 5 genes associated with muscle metabolism and development: *SLCO3A1*, *ATP6V0D1*, *SLC2A1*, *GPC4*, and *RASD2*. On blood, among the top 10 DEG, we identified genes up-regulated in the REST after weaning related to response to stress, such as

SOD3 and *INO80D*, and down-regulated in the REST after vaccination related to immune response, such as *OASL, KLRF1*, and *LOC104968634.* Among the DEG identified in both blood and muscle, *VAT1, CABLES1, SLC20A2, ILF3,* and *QDPR* were down-regulated in the muscle and up-regulated in blood, and *SPAG17* and *LOC107131247* were down-regulated in both.

Conclusion

In conclusion, maternal energy restriction during late gestation may limit the expression of genes in the muscle and increase expression in the blood of the calves. In addition, enrichment analysis showed that a short-term maternal energy restriction during pregnancy affects the expression of genes related to muscle carbohydrate metabolic process, and muscle contraction in muscle, as well as immunity and stress response in the blood. Therefore, alterations in the intra-uterine environment can modify prenatal development with lasting consequences through adult life.

Keywords: RNA-seq, fetal development, fetal programming, gene expression

Background

Maternal nutrition has been identified as one of the main factors affecting intra-uterine environment [1]. There is an increase in nutritional requirements of beef cows during late gestation [2], and if not met, the limited amount of nutrients available for optimum fetal development may affect prenatal physiological functions and, consequently, result in impaired post-natal growth and performance of the offspring [3,4]. Therefore, the last trimester of gestation is a critical period for fetal growth [3].

Metabolizable energy restriction has been shown to down-regulate the mitogenic responses of T lymphocytes in beef calves [4]. Prenatal programming of physiological systems can alter the growth and function of organs and pathology into adulthood [5]. In other words, nutritional deficiency may impact the regulation of genes associated with myogenesis and immune response in the fetus, which can have long-term effects on progeny growth performance [3].

The deposition of skeletal muscle has significant implications to the animal meat-production systems. Muscle fibers are formed mainly during mid-gestation, and the definitive number of fibers is determined before birth [6]. On the other hand, fiber hypertrophy occurs during late gestation and continues following birth [6]. In addition, since a well-developed immune system protects the animal from pathogens and other stressors, healthy animals present better performance compared to immune compromised animals during the growth and development [7]. Moreover, the prenatal programming of the immune system would give rise to changes that persist over the course of the animal's life [8].

Transcriptome studies have shown changes in fetal growth and immune factors, especially during the final trimester of gestation. Previous studies have reported differentially expressed genes (DEG) in the muscle of fetuses as an effect of maternal diet during pregnancy based on different energy sources, which ultimately change the metabolalizable energy intake [9,10]. For example, decrease in expression of genes involved in muscle synthesis and differentiation, tissue and organ development, chromatin biology, and metabolic processes have been reported in calves as an effect of maternal diet during pregnancy based on corn compared with a diet based on alfalfa haylage, and dried corn distillers grains [9]. In addition, O'Loughlin et al. [11] identified DEG involved in cytokine

signaling, transmembrane transport, hemostasis and G-protein-coupled receptor signaling as a response to weaning stress in calves.

The preconditioning phase is a critical period for beef calves, since animals pass through stressful processes such as vaccination, weaning, and diet changes [12,13]. As a response, there is a mobilization of protein from muscle [14] to increase the synthesis of proteins and cells involved in the immune system [15]. Consequently, growth performance is compromised due to limited availability of nutrients for skeletal muscle development. Also during this period, there is a decrease in energy and protein intake [16], which may reduce the immune capacity of the animal and, consequently, impact vaccination response [13].

We hypothesized that maternal energy restriction would cause changes in the expression of genes related to muscle development and immune response in preconditioned beef calves born from cows that experienced short-term energy restriction (30% of total energy requirements) during the last 40 d of gestation compared to unrestricted controls. Therefore, the objectives of this work were to (1) identify DEG in the muscle and blood of beef calves born from cows with and without energy restriction, (2) to assess the biological relevance of DEG, and (3) to investigate the relationships of DEG through gene networks.

Materials and methods

Animals and Diets

Thirty recipients multiparous, nonlactating, pregnant spring-calving Angus cows with average body weight 631 ± 15 kg, 5.2 ± 0.98 years of age, and body condition score of 6.3 ± 0.12 were used in this study. Cows were sired by two sires, and forty days before the expected calving date, cows were randomly assigned to one of two isonitrogenous diets: the control group (CTRL) and the restricted group (REST). Animals received total-mixed diets

formulated to provide 100% (CTRL) or 70% (REST) of the daily net energy requirement for maintenance of a 630 kg beef cow at 8 months of gestation [17]. Animals were randomly assigned to pens according to the treatment (n = 5 pens/treatment). Immediately after calving, cow–calf pairs were transferred to 1 of 6 tall fescue pastures with free choice access to water and a complete mineral mix. Cows received dietary treatments for 40 ± 5.08 days. All male calves were castrated by banding immediately after birth. Cows and calves were managed as a single group and rotated among pastures monthly from calving until weaning (approximately 227 days of age). From weaning (day 0) until 40 days post weaning (dpw), calves were assigned to a preconditioning period. Additional detailed information regarding the nutritional information of the diets, feeding strategies, and the design of the study have been previously described in Moriel et al. [18] .

Preconditioning

At weaning (0 dpw), calves were individually treated with doramectin for internal and external parasites (5 mL subcutaneous; Dectomax injectable; Zoetis Inc., Kalamazoo, MI). At 8 dpw, calves were vaccinated against infectious bovine rhinotracheitis virus, bovine viral diarrhea virus types 1a and 2, parainfuenza 3 virus, *Mannheimia haemolytica* (2 mL subcutaneous; Bovi Shield Gold One Shot; Zoetis Inc.), and Clostridium spp. (2 mL subcutaneous; Ultrabac 7; Zoetis Inc.). At 21 dpw, calves received 2 mL subcutaneous boosters of Bovi Shield Gold 5 (Zoetis Inc.) and Ultrabac 7. This vaccination protocol was used to replicate the standard protocol used by the local preconditioning alliance (Mountain Cattle Alliance, Canton, NC) [19,20].

Tissue Collection

At 21 dpw, a biopsy of the skeletal muscle *Longissimus dorsi* was performed in all calves (12 steers and 12 heifers) at the level of the pelvis between the iliac, coxal and ischial tuberosity to obtain a minimum sample of tissue for subsequent gene expression analysis. Muscle biopsies were placed into a 2 mL Cryovial tube containing RNAlater (Ambion Inc., Austin, TX, USA) and stored in a −20°C freezer for subsequent laboratory analysis. In addition, blood samples (10 mL) were collected from all steers via jugular venipuncture into Tempus Blood RNA Tubes (Life Technologies, Carlsbad, CA, USA) at 0, 3, 6, and 15 dpw for subsequent gene expression analysis. Blood samples were immediately put on ice and stored at −80°C until later laboratory analysis. Although 30 cows were initially used in the trial, subsequent analyses were performed on a subset of samples because of costs. Thus, a random sample of 24 calves (12 steers and 12 heifers) and 12 steers (one from each pen) were used for muscle and blood analyses, respectively. Since, four time-points were sampled for blood, we opted to analyze just one sex to avoid increasing complexity of the statistical models and excessive costs.

RNA Extraction, Sequencing, and Bioinformatics

Total RNA from muscle and blood samples were extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Germantown, MD, USA) and Tempus^{TM} RNA isolation kit (Applied Biosystems, Foster City, CA, USA), respectively. The RNA quantity and quality were determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). One muscle and one blood samples were excluded from subsequent analysis due to low RNA integrity number (RIN) score (RIN < 8). All remaining samples were sent out to the Genomic Sciences Laboratory (North Carolina State University, Raleigh, NC, USA) for

library construction and RNA-sequencing. Sequencing was performed on an Illumina NextSeq 500 instrument (Illumina, Inc., San Diego, USA), generating 75 bp paired-reads and 150 bp single-end reads for muscle and blood, respectively. A total of 2 and 3 flow cells were used, respectively, for muscle and blood samples, with approximately equal representation of treatments between flow cells.

Quality of raw reads were evaluated with FastQC [21]. Sequence reads for each sample were mapped to the *Bos taurus* UMD3.1 reference genome using Bowtie2 [22]. The number of counts for each sample was obtained with the *Subread* package from SourceForge [23]. A total of 2,076,680,240 paired-ends and 1,353,110,288 single-ends reads were generated for muscle and blood samples, respectively, with an average of 90,290,445 and 28,789,580 reads/sample, respectively. Reads were mapped to a total of 77.43% (19,045) and 78.59% (19,331) genes for muscle and blood, respectively, from a total of 24,596 annotated genes in the UMD3.1 *Bos taurus* reference genome. Genes with less than four times the number of counts as samples were eliminated to avoid low counts across multiple samples, resulting in a final set of 15,255 and 13,512 genes for muscle and blood, respectively. For normalization of the data, we used Trimmed Mean of M-values (TMM) to calculate the normalization factors (*TCC* package [24]). The normalized library size was obtained by dividing the total library size by the normalized factors.

Statistical Analyses

Gene expression data was analyzed with a negative binomial model and a log link function [25]. Different models were tested for each tissue. For muscle, a negative binomial function was used in the following model:

$$Y_{ijklmn} = \mu + D_i + S_j + (D * S)_{ij} + B_k + P_l + \beta_1\left(R_{ijklmn} - \bar{R}\right) + \beta_2\left(DOT_{ijklmn} - \overline{DOT}\right) + SS_n + log\left(L_{ijklmn}\right)$$

where, Y_{ijklmn} is the raw number of counts; μ is the intercept, D_i is the fixed-effect of the ith Diet; S_j is the fixed effect of the jth Sex; B_k is the fixed effect of the kth Batch (sequencing lane); P_l is the fixed effect of the lth Pen; β_1 is the partial regression coefficient for the covariate RIN scores (R); β_2 is the partial regression coefficient for Days on Dietary Treatment (DOT); SS_n is the fixed effect of the nth Service Sire; and L_{ijklmn} is the TMM-normalized library size, used as an offset. In addition to this model, 3 other reduced models were tested, in which the effects of pen and/or the interaction were removed. All models were used for each of the 15,255 genes, and the final model for each gene was chosen based on Akaike information criterion (AIC).

For the gene expression analysis of blood samples, a negative binomial function was used in the following model:

$$Y_{ijklmn} = \mu + D_i + T_j + (D * T)_{ij} + B_k + \beta_1\left(R_{ijklmn} - \bar{R}\right) + \beta_2\left(DOT_{ijklmn} - \overline{DOT}\right) + SS_n + log\left(L_{ijklmn}\right)$$

where, Y, μ is the intercept, and D, B, R, L, DOT, and SS are as previously defined. T_j is the fixed effect of the jth Time. A reduced model without interaction was tested, and analyses were performed using three covariance structures for the residuals: first-order autoregressive, compound symmetry, and independent residuals. Therefore, 6 models were tested for each of the 13,512 genes, and the final model for each gene was selected based on AIC. Satterthwaite approximation to account for sample variances was used to determine the denominator

degrees of freedom. The dispersion parameter of the model was calculated for each gene squeezing towards a global dispersion with an approach assuming mean and variance related by $\sigma^2 = \mu + a\mu^2$ [26], where a is a proportionality constant, using the package e*dgeR* in R software [27].

Additional contrasts were constructed in order to answer biologically relevant questions in the analysis of blood samples. Contrasts were developed for both the interaction effect (diet-by-time) and the main effect of time. For the main effect of time, two contrasts with 1-degree-of-freedom (df) were constructed: (1) to test the effect of vaccination (i.e. average of days 0, 3, and 6, versus day 15), and (2) to evaluate the acute response to weaning (i.e. day 0 versus average of days 3 and 6). For the interaction effect, these same contrasts were constructed, but accommodating their interaction with diet.

False-discovery rate (FDR [28]) was used to adjust the *P*-values (*q*-values) of model terms due to multiple testing. Significant DEG were identified at *q*-value ≤ 0.05 for all analyses. All data were analyzed using the GLIMMIX procedure of SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

Functional Annotation Analysis

The enrichment of Gene Ontology (GO) terms associated with DEG was analyzed using PANTHER Enrichment Analysis [29]. Different DEG lists were created based on the significance (*q*-value < 0.05) of effects in the model. For the muscle data, analyses were performed separately for DEG based on diet and sex. For blood, we performed 3 analyses, one based on the effect of diet, and two based on the previously described contrasts (effect of vaccination and weaning). The *Bos taurus* genome was used as the background list. Biological Processes was considered significant at *P*-value < 0.05.

Gene-Network Analysis

Two gene networks were constructed for the effect of diet: one using the most significant top 20 DEG in the muscle, and another using the top 10 DEG for the effect of diet in muscle associated with the top 10 DEG in blood. Partial correlation networks were constructed using gene counts pre-adjusted for all effects in the model, with the exception of diet (the effect of interest). In other words, the data used in this analysis, for each individual, represented the sum of the estimated diet effect and its residual. For the network including DEG from muscle and blood tissues, only data from steers were used as only male offspring had data using both tissues. Connections between genes (i.e. edges) were included when a pair of genes showed a partial correlation greater than |0.5|. The correlation matrix and gene-networks were constructed using the *ppcor* [30] and *qgraph* [31] packages in R software [27].

Results

DEG Identification

The number of DEG identified in this study is shown in Figure 2.1. A total of 160, 164, and 346 DEG (q-value < 0.05) were identified in the muscle for the effects of diet, sex, and diet-by-sex interaction, respectively (Figure 2.1A). For blood, 452, 1392, and 155 DEG (q-value < 0.05) were identified for the effects of diet, time, and diet-by-time interaction, respectively (Figure 2.1B). For the contrasts in blood tissue, there were 101 and 47 DEG (q-value < 0.05) for Weaning and Vaccination, respectively, based on the diet-by-time interaction effect (q-value < 0.05). For the main effect of Time (q-value < 0.05), there were 893 and 473 DEG (P-value < 0.05) for Weaning and Vaccination contrasts, respectively.

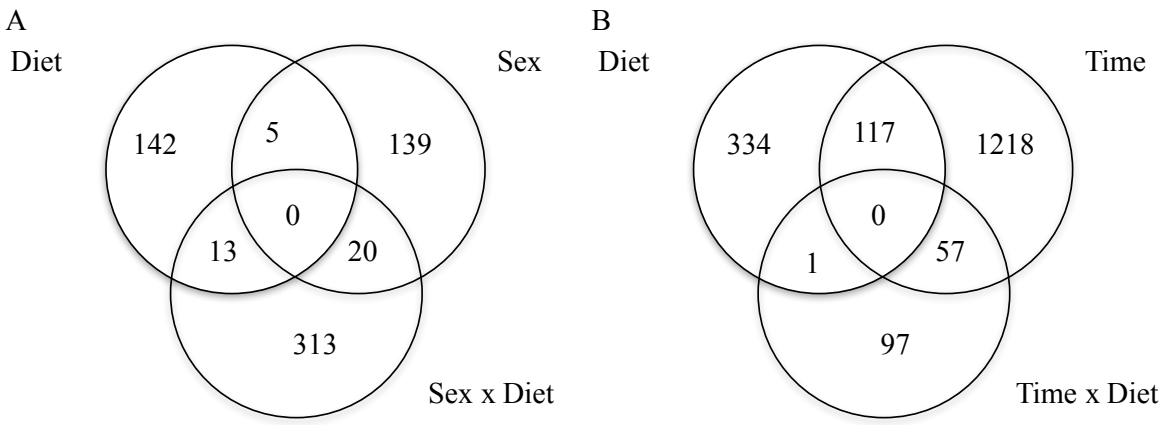

Figure 2.1 *Venn Diagram. Number of differentially expressed genes (DEG; q-value < 0.05) for the effects of diet, sex, and diet-by-sex for muscle (A), and diet, sex and diet-by-time for blood (B).*

Volcano plots for the effect of diet are shown in Figure 2.2. For the effect of sex in the muscle, there was a greater number of up-regulated genes for females (117) compared with males (47) (Figure 2.2A). There was a greater number of down-regulated (131) genes in the REST compared to up-regulated (29) in the muscle tissue (Figure 2.2B). In contrast, there was a much greater number of up-regulated genes in the REST (410) compared to down-regulated (42) in the blood (Figure 2.2C).

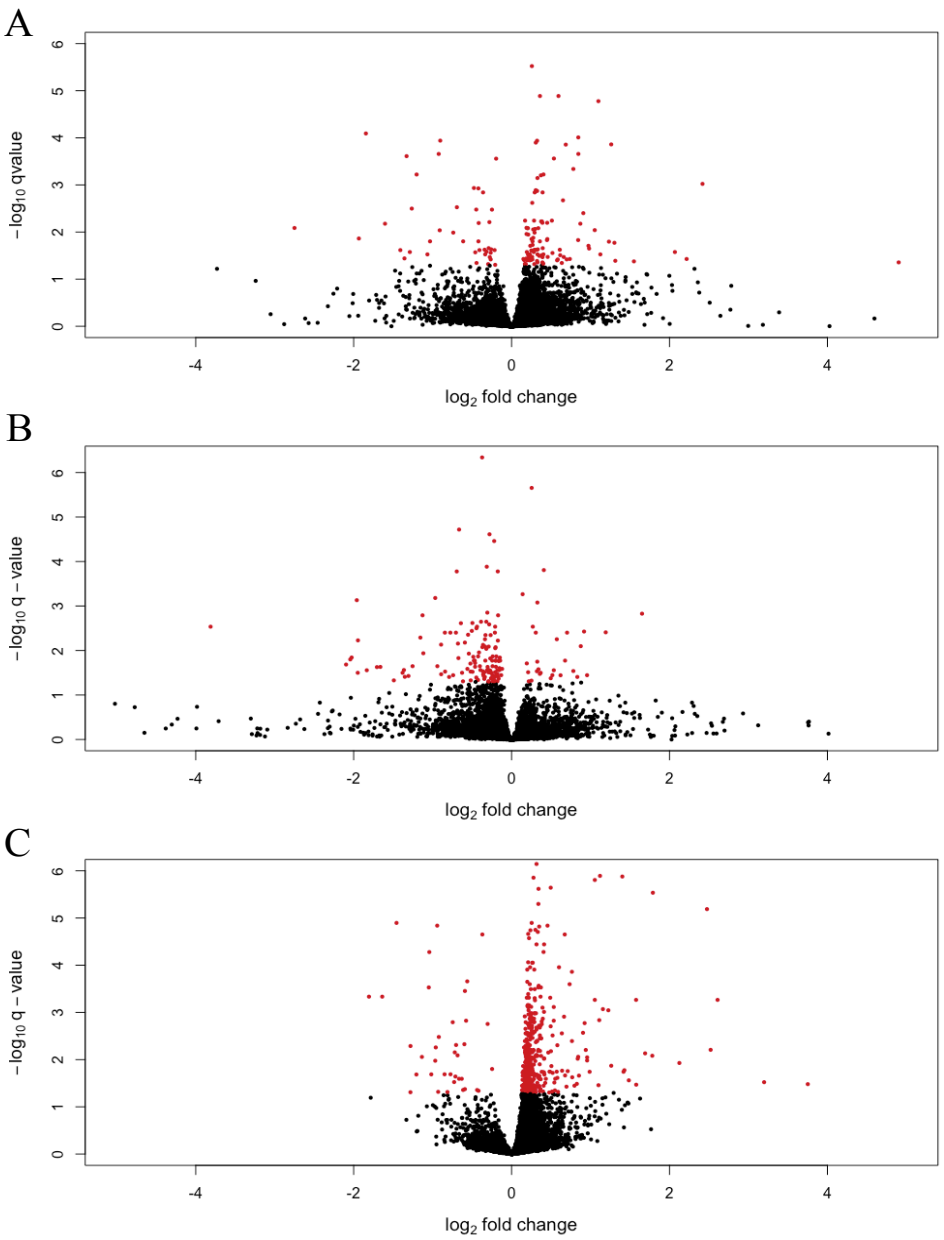

Figure 2.2 *Volcano plots for the effect of sex (A) and diet (B) in the muscle tissue, and for the effect of diet blood (C) tissue. The Y-axis shows the –log10 q-values for the effect of diet, whereas the X-axis represents the log2 fold change, with negative and positive values representing the down- and up-regulation of DEG in the restriction-enetgy group (REST), respectively. Genes with significant (q-value < 0.05) effect of diet are highlighted in red.*

Enrichment Analysis in the Muscle

The results of the enrichment analysis for diet and sex are presented in Table 2.1. Results based on diet ($P < 0.05$) showed genes involved in muscle metabolism, i.e. *positive regulation of actin cytoskeleton reorganization* (GO:2000251)*, positive regulation of collagen biosynthetic process* (GO:0032967), *adipose tissue development* (GO:0060612) and *positive regulation of collagen metabolic process* (GO:0010714); and nervous system metabolism, i.e *regulation of neuron differentiation* (GO:0045664), *regulation of neurogenesis* (GO:0050767), *regulation of nervous system development* (GO:0051960), and *negative regulation of neuron projection development* (GO:0010977). Enrichment analyses for the effect of sex showed that DEG were enriched for categories related to nucleic acid metabolic process, such as *histone H3-K27 demethylation* (GO:0071557), *DNA-dependent DNA replication* (GO:0006261), *DNA replication initiation* (GO:0006270), and *DNA repair* (GO:0006281); and metabolism of carbohydrate and muscle, such as *positive regulation of glycoprotein biosynthetic process* (GO:0010560), *regulation of skeletal muscle contraction* (GO:0014819), and *regulation of glycogen metabolic process* (GO:0070873). Additional results ($P < 0.05$) including biological function GO terms for the effects of diet and sex can be seen in APPENDIX.

Table 2.1 Enrichment analysis showing top 20 overrepresented[1] Biological Processes for Differentially Expressed Genes[2] for the effects of diet and sex in the muscle tissue

Biological Process	# genes	FE	*P*-value
Diet			
Positive regulation of transport (GO:0051050)	14	2.92	3.81E-04
Regulation of neuron projection development (GO:0010975)	8	4.27	7.09E-04
Negative regulation of myeloid cell differentiation (GO:0045638)	4	9.56	1.02E-03
Regulation of neuron differentiation (GO:0045664)	9	3.32	1.86E-03

Table 2.1 continued

Biological Process	# genes	FE	*P*-value
Positive regulation of blood vessel endothelial cell migration (GO:0043536)	3	13.21	1.90E-03
Regulation of transport (GO:0051049)	20	2.08	2.13E-03
Regulation of cell morphogenesis involved in differentiation (GO:0010769)	6	4.55	2.44E-03
Regulation of cell morphogenesis (GO:0022604)	8	3.33	3.28E-03
Regulation of intracellular transport (GO:0032386)	7	3.55	4.16E-03
Regulation of protein targeting (GO:1903533)	4	6.33	4.26E-03
Membrane depolarization (GO:0051899)	3	9.4	4.70E-03
Positive regulation of actin cytoskeleton reorganization (GO:2000251)	2	21.68	4.88E-03
Peptidyl-proline hydroxylation (GO:0019511)	2	21.68	4.88E-03
Regulation of mitochondrion organization (GO:0010821)	5	4.49	5.87E-03
Negative regulation of hemopoiesis (GO:1903707)	4	5.75	5.90E-03
Cell surface receptor signaling pathway (GO:0007166)	20	1.87	6.52E-03
Regulation of nervous system development (GO:0051960)	10	2.55	6.61E-03
Regulation of neurogenesis (GO:0050767)	9	2.68	7.28E-03
Regulation of localization (GO:0032879)	25	1.74	7.58E-03
Regulation of plasma membrane bounded cell projection organization (GO:0120035)	8	2.86	7.79E-03
Sex			
Kinetochore assembly (GO:0051382)	3	52.18	5.64E-05
Kinetochore organization (GO:0051383)	3	34.79	1.52E-04
Positive regulation of glycoprotein biosynthetic process (GO:0010560)	3	34.79	1.52E-04
Positive regulation of glycoprotein metabolic process (GO:1903020)	3	29.82	2.25E-04
Regulation of glucose metabolic process (GO:0010906)	5	9.53	2.41E-04
Chromosome organization (GO:0051276)	17	2.59	3.56E-04
Centromere complex assembly (GO:0034508)	3	21.97	4.97E-04
Establishment of melanosome localization (GO:0032401)	3	20.87	5.68E-04
Regulation of cellular carbohydrate metabolic process (GO:0010675)	5	7.65	6.29E-04

Table 2.1 continued

Biological Process	# genes	FE	*P*-value
Establishment of pigment granule localization (GO:0051905)	3	19.88	6.46E-04
Melanosome localization (GO:0032400)	3	19.88	6.46E-04
Pigment granule localization (GO:0051875)	3	18.98	7.30E-04
Histone H3-K27 demethylation (GO:0071557)	2	55.66	1.04E-03
ATP hydrolysis coupled transmembrane transport (GO:0090662)	3	16.06	1.14E-03
ATP hydrolysis coupled proton transport (GO:0015991)	3	16.06	1.14E-03
Regulation of carbohydrate metabolic process (GO:0006109)	5	6.5	1.26E-03
Cellular macromolecular complex assembly (GO:0034622)	14	2.57	1.32E-03
Energy coupled proton transmembrane transport, against electrochemical gradient (GO:0015988)	3	14.91	1.38E-03
Positive regulation of sodium ion transmembrane transporter activity (GO:2000651)	2	39.76	1.76E-03
Regulation of skeletal muscle contraction (GO:0014819)	2	34.79	2.19E-03

[1]*P*-value < 0.05;
[2]*q*-value < 0.05;
FE = fold enrichment

Major DEG in the Skeletal Muscle

The most top 10 significant up- and down-regulated DEG in the REST are summarized in Table 2.2. Of these, the most significant up- and down-regulated gene in the REST were *SLCO3A1* and *ETNPPL*, with $\log_2$FC of -0.37 ± 0.06 (*q*-value = 4.58E-07) and 0.41 ± 0.08 (*q*-value = 1.56E-04), respectively. The largest |$\log_2$FC| was observed for *GTF2A1L*, with -6.01 ± 1.67 (*q*-value = 3.60E-02).

Table 2.2 Top 10 up- and down-regulated most differentially expressed genes[1,2] in the muscle tissue for the energy-restricted (REST) group

Gene symbol	Gene name	Biological function	Log_2 FC ± SE	*q*-value
SLCO3A1	Solute carrier organic anion transporter family member 3A1	Thyroid hormone membrane transporter involved in satellite cell differentiation [32]	-0.37 ± 0.06	4.58E-07
LAT	Inker for activation of T cells	T cell activation [33]	-0.66 ± 0.11	1.91E-05
MLX	MAX dimerization protein	Control cell cycle [34]	-0.28 ± 0.05	2.44E-05
TSPAN7	Tetraspanin 7	Maturation of glutamatergic synapses [35]	-0.31 ± 0.06	1.31E-04
ATP6V0D1	ATPase H+ transporting V0 subunit d1	Involved in *oxidative* phosphorylation [36]	-0.17 ± 0.03	1.67E-04
SLC2A1	Solute carrier family 2 member 1	Glucose transporter [37]	-0.69 ± 0.13	1.67E-04
RASL10B	RAS like family 10 member B	Potential tumor suppression [38]	-0.97 ± 0.19	6.60E-04
GPC4	Glypican 4	Involved in cell proliferation [39]	-0.31 ± 0.06	1.41E-03
RTF2	Replication termination factor 2	Riboflavin transporters [40]	-0.17 ± 0.04	1.62E-03
RASD2	RASD family member 2	Involved in cellular movement and cell cycle [41]	-0.39 ± 0.08	2.25E-03
ETNPPL	Ethanolamine-phosphate phospholyase	Associated to fatty acid and lipid metabolic process [42]	0.41 ± 0.08	1.56E-04
TRAPPC6B	Trafficking protein particle complex 6B	Vesicle transport [43]	0.14 ± 0.03	5.42E-04
SMIM11A	Small integral membrane protein 11A		0.33 ± 0.07	8.36E-04

Table 2.2 continued

Gene symbol	Gene name	Biological function	Log_2 FC ± SE	*q*-value
TMCC2	Transmembrane and coiled-coil domain family 2	Play a role in neurodegeneration [44]	0.27 ± 0.06	2.91E-03
SYT3	Synaptotagmin 3	Involved in glucose metabolism [45]	0.92 ± 0.20	3.75E-03
SPATA	Spermatogenesis associated 1	Involved in male fertility [46]	1.19 ± 0.27	3.91E-03
AQP4	Aquaporin 4	Water channels [47]	0.70 ± 0.16	3.97E-03
ETFRF1	Electron transfer flavoprotein regulatory factor 1	Involved in fatty acid oxidation [48]	0.31 ± 0.07	3.97E-03
CHN1	Chimerin 1	GTPase-activating protein [49]	0.57 ± 0.13	5.59E-03
FZD5	Frizzled class receptor 5	Wnt/Wingless (Wg) signals [50]	0.88 ± 0.21	8.00E-03

[1]*q*-value < 0.05;
[2]Up- and down-regulated in the groups are represented by positive and negative log_2FC estimates, respectively;
Biological Function based on cited reference;
SE = Standard Error

The most significant top 10 DEG for the effect of sex are summarized in Table 2.3. The most significant gene up-regulated in males was *RIMS1* with log_2FC of -2.83 ± 0.24 (*q*-value = < 1.00E-13), and in females was *SYAP1* with 0.35 ± 0.04 (*q*-value = < 1.00E-13). The largest |log_2FC| was observed for LOC107131189 with -6.64 ± 0.24 (*q*-value =< 1.00E-13).

Table 2.3 Top 10 most differentially expressed genes[1,2] in the muscle tissue for the effect of sex.

Gene symbol	Gene name	Biological function	Log_2 FC ± SE	*q*-value
RIMS1	Regulating synaptic membrane exocytosis 1	Maintenance of normal synaptic function [51]	-2.83 ± 0.24	< 1.00E-13

Table 2.3 continued

Gene symbol	Gene name	Biological function	Log_2 FC ± SE	*q*-value
LOC107131189	Eukaryotic translation initiation factor 2 subunit 3, Y-linked-like		-6.64 ± 0.13	< 1.00E-13
LOC107131205	Lysine-specific demethylase 6A-like	Antioxidant [52]	-3.86 ± 0.35	< 1.00E-13
CD3G	CD3g molecule	T-cell receptor [53]	-1.85 ± 0.17	8.08E-05
ADAM1B	A disintegrin and metallopeptidase domain 1b	Involved in male fertilization [54]	-0.90 ± 0.18	1.15E-04
ANOS1	Anosmin 1	Cell adhesion [55]	-0.92 ± 0.26	2.20E-04
ACTL8	Actin like 8		-1.33 ± 0.04	2.45E-04
ARID1B	AT-rich interaction domain 1B	Regulation of cell cycle [56]	-0.20 ± 0.25	2.77E-04
GCGR	Glucagon receptor	Glucagon receptor [57]	-1.20 ± 0.09	6.00E-04
ZBED1	Zinc finger BED-type containing 1	Associated with cell proliferation [58]	-0.42 ± 0.24	1.22E-03
SYAP1	Synapse associated protein 1		0.35 ± 0.04	< 1.00E-13
KDM6A	Lysine demethylase 6A	Demethylase [59]	0.74 ± 0.07	< 1.00E-13
EIF2S3	Eukaryotic translation initiation factor 2 subunit gamma	Play a role in pituitary development and insulin secretion [60]	0.57 ± 0.05	< 1.00E-13
TXLNG	Taxilin gamma		0.95 ± 0.13	8.48E-10
ZRSR2	Zinc finger CCCH-type, RNA binding motif and serine/arginine rich 2		0.69 ± 0.10	4.27E-08
SRD5A3	Steroid 5 alpha-reductase 3	Produces steroid hormones [61]	0.46 ± 0.07	6.49E-08

Table 2.3 continued

Gene symbol	Gene name	Biological function	Log_2 FC ± SE	*q*-value
TMCC2	Transmembrane and coiled-coil domain family 2		0.36 ± 0.06	1.30E-05
FAAP24	Fanconi anemia core complex associated protein	DNA damage response [62]	1.10 ± 0.20	1.67E-05
USP9X	Ubiquitin specific peptidase 9, X-linked	TGF-β signaling [63]	0.32 ± 0.06	1.15E-04
ICMT	Isoprenylcysteine carboxyl methyltransferase	Methyltransferase [64]	0.31 ± 0.06	1.26E-04

[1]*q*-value < 0.05;
[2]Negative and positive log_2FC estimates represents up-regulated in males and females, respectively;
Biological Function based on cited reference;
SE = Standard Error

Enrichment Analysis in the Blood

The enrichment analyses for the different sets of DEG in the blood are presented in Table 2.4. Results based on diet showed general metabolic functions ($P < 0.01$), such as t*ranslation* (GO:0006412), *rRNA metabolic process* (GO:0016072), and *biosynthetic process* (GO:0009058) in addition to carbohydrate metabolism such as *regulation of carbohydrate metabolic process* (GO:0006109) and *glycogen metabolic process* (GO:0005977). In contrast, for results based on biologically relevant contrasts, there was an overrepresentation of biological processes related to immune response for the effect of vaccination, such as *immune system process* (GO:0002376), *regulation of response to stimulus* (GO:0048583), and *positive regulation of response to stimulus* (GO:0048584), in addition to other general biological processes, such as *localization* (GO:0051179), and *transport* (GO:0006810). For the effect of weaning, we found GO terms associated with response to stress and the immune

system, i.e. *cellular response to DNA damage stimulus* (GO:0006974)*, response to stress* (GO:0006950)*, regulation of lymphocyte activation* (GO:0051249) and *response to interleukin-4* (GO:0070670). Additional enrichment analysis results ($P < 0.05$) including GO terms for the effects of time (weaning and vaccination) can be seen in the APPENDIX.

Table 2.4 Enrichment analysis showing the top 20 overrepresented[1] Biological Process for Differentially Expressed Genes[2] for diet and biologically relevant contrasts in the blood tissue (effect of weaning and vaccination)

Biological Process	# genes	FE	P-value
Diet			
Translation (GO:0006412)	22	4.3	3.42E-08
Cellular component biogenesis (GO:0044085)	36	2.08	6.01E-05
rRNA metabolic process (GO:0016072)	10	3.77	5.20E-04
Cellular component organization or biogenesis (GO:0071840)	65	1.48	1.68E-03
Biosynthetic process (GO:0009058)	55	1.49	4.07E-03
Organelle organization (GO:0006996)	39	1.51	1.36E-02
Regulation of carbohydrate metabolic process (GO:0006109)	3	6.54	1.37E-02
Transcription initiation from RNA polymerase II promoter (GO:0006367)	4	3.81	2.47E-02
Protein localization (GO:0008104)	18	1.72	2.71E-02
Protein targeting (GO:0006605)	8	2.3	2.73E-02
Primary metabolic process (GO:0044238)	119	1.19	3.26E-02
Cellular component organization (GO:0016043)	54	1.32	3.77E-02
Glycogen metabolic process (GO:0005977)	3	4.03	4.38E-02
Weaning			
Translation (GO:0006412)	101	5.84	6.58E-42
Peptide biosynthetic process (GO:0043043)	101	5.64	8.81E-41
Amide biosynthetic process (GO:0043604)	103	5.09	3.67E-38
Peptide metabolic process (GO:0006518)	103	4.8	3.40E-36
Cellular amide metabolic process (GO:0043603)	106	4.08	7.70E-32
Cellular macromolecule biosynthetic process (GO:0034645)	181	2.21	4.73E-23
Macromolecule biosynthetic process (GO:0009059)	181	2.17	1.99E-22
Organonitrogen compound biosynthetic process (GO:1901566)	125	2.68	4.22E-22
Cellular nitrogen compound biosynthetic process (GO:0044271)	165	2.11	4.11E-19
Cellular macromolecule metabolic process (GO:0044260)	293	1.62	1.85E-18
Cellular protein metabolic process (GO:0044267)	213	1.84	3.63E-18
Organic substance biosynthetic process (GO:1901576)	203	1.8	2.37E-16

Table 2.4 continued

Biological Process	# genes	FE	P-value
Cellular nitrogen compound metabolic process (GO:0034641)	232	1.7	3.41E-16
Cellular biosynthetic process (GO:0044249)	198	1.8	9.94E-16
Biosynthetic process (GO:0009058)	204	1.76	1.74E-15
Protein metabolic process (GO:0019538)	232	1.64	1.84E-14
Gene expression (GO:0010467)	168	1.85	3.42E-14
Organelle organization (GO:0006996)	178	1.74	6.55E-13
Macromolecule metabolic process (GO:0043170)	320	1.42	2.33E-12
Cellular component organization or biogenesis (GO:0071840)	252	1.52	2.74E-12
Ribosome biogenesis (GO:0042254)	37	3.65	1.55E-10
Vaccination			
Localization (GO:0051179)	141	1.61	3.46E-09
Establishment of localization (GO:0051234)	110	1.6	6.83E-07
Immune system process (GO:0002376)	58	1.93	2.65E-06
Transport (GO:0006810)	104	1.57	3.50E-06
Positive regulation of GTPase activity (GO:0043547)	22	3.18	4.20E-06
Cellular response to chemical stimulus (GO:0070887)	67	1.75	1.25E-05
Regulation of GTPase activity (GO:0043087)	23	2.87	1.27E-05
Regulation of response to stimulus (GO:0048583)	97	1.54	2.14E-05
Positive regulation of response to stimulus (GO:0048584)	56	1.78	3.82E-05
Positive regulation of hydrolase activity (GO:0051345)	28	2.4	3.93E-05
Cellular response to organic substance (GO:0071310)	55	1.77	5.19E-05
Cell differentiation (GO:0030154)	84	1.53	8.59E-05
Regulation of response to external stimulus (GO:0032101)	26	2.35	9.76E-05
Transition metal ion transport (GO:0000041)	9	5.07	1.32E-04
Cellular homeostasis (GO:0019725)	28	2.24	1.36E-04
Developmental process (GO:0032502)	118	1.39	1.65E-04
Regulation of hydrolase activity (GO:0051336)	40	1.92	1.71E-04
Myeloid leukocyte activation (GO:0002274)	8	5.47	1.93E-04
Positive regulation of response to external stimulus (GO:0032103)	14	3.22	2.06E-04
Regulation of signaling (GO:0023051)	83	1.5	2.22E-04
Regulation of signal transduction (GO:0009966)	77	1.53	2.22E-04

[1] *P*-value < 0.05;
[2] *q*-value < 0.05;
FE = fold enrichment

Major DEG in Blood Tissue

The effect of the interaction diet-by-time was analyzed based on biologically relevant contrasts. The top 10 most significant DEG for the effect of interaction diet-by-weaning and diet-by-vaccination are summarized in Table 2.5. Of these, the most significant gene for the effect of the interaction diet-by-weaning was *USF3* (*q*-value = 1.13E-04) and for the effect of diet-by-vaccination was *LOC104968634* (*q*-value = 6.62E-04). In addition, we found genes related to immune system function and response to stress, such as *KLRK1* [65] and *INO80D* [66], down and up-regulated, respectively, in the REST after weaning. When we looked at the effect of the interaction diet-by-vaccination, we found genes involved in immune response being down-regulated in the REST after vaccination, including *OASL* [67], *KLRF1* [65], and *LOC104968634* [68].

Table 2.5 Top 10 Differentially Expressed Genes[1] in the blood for biologically relevant contrasts

Gene symbol	Gene name	Biological function	*q*-value
DEG for effect of diet-by-weaning interaction			
USF3	Upstream trasnscription factor family member 3	*USF3*	1.13E-04
STARD6	StAR related lipid transfer domain containing 6	Structure and lipid transport mechanism [69]	3.10E-04
PHC3	Polyhomeotic homolog 3		3.17E-04
BRMS1L	Breast cancer metastasis-suppressor 1 like	Metastasis suppression [70]	4.87E-04
MPV17L	MPV17L mitochondrial inner membrane protein like	Up- or down-regulation of the genes of antioxidant enzymes [71]	4.92E-04
NBEAL1	Neurobeachin like 1	Vesicle trafficking, membrane dynamics and receptor signaling [72]	7.31E-04
C5H12orf4	Chromosome 5 open reading frame, human C12orf4		7.59E-04

Table 2.5 continued

Gene symbol	Gene name	Biological function	*q*-value
INO80D	INO80 complex subunit D	DNA damage responses [73]	8.00E-04
TIGD3	Tigger transposable element derived 3		8.94E-04
KLRK1	Killer cell lectin like receptor F1	Stimulates natural killer (NK) cells cytoxicity and cytokine release [65]	9.05E-04
DEG for effect of diet-by-vaccination interaction			
LOC104968634	Antimicrobial peptide NK-lysin-like	Antimicrobial activity [68]	6.62E-04
GPR161	G protein-coupled receptor 161	Involved in neural tube development [74]	2.13E-03
KLRF1	Killer cell lectin like receptor F1	Stimulates NK cells cytoxicity and cytokine release [65]	2.20E-03
PMCH	Pro-melanin concentrating hormone	Feed intake control [75]	2.81E-03
PIK3C2G	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma	Control cell proliferation [76]	3.15E-03
KRT24	Keratin 24		3.64E-03
PRRT3	Proline rich transmembrane protein 3	Protein binding [77]	3.74E-03
QRICH2	Glutamine rich 2	Cupper regulation [78]	4.18E-03
HIST2H2AB	Histone cluster 2 H2A family member B	Play a central role in transcription regulation, DNA repair and DNA replication [79]	4.22E-03
OASL	2'-5'-oligoadenylate synthetase like	Response to viral infections [67]	4.73E-03

[1]*P*-value < 0.05 for significant (*q*-value < 0.05) effects of diet-by-time interaction or time; Biological Function based on cited reference;

Partial Correlation Network in the Muscle and Blood

The partial correlation network using the 20 most DEG (q-value < 0.05) is depicted in Figure 2.3. On average, each gene was connected to 9.8 genes, with *ATP6V0D1* and *TMCC2* showing the greatest number of connections (15 genes) between them, and *MATK* the least (2 genes).

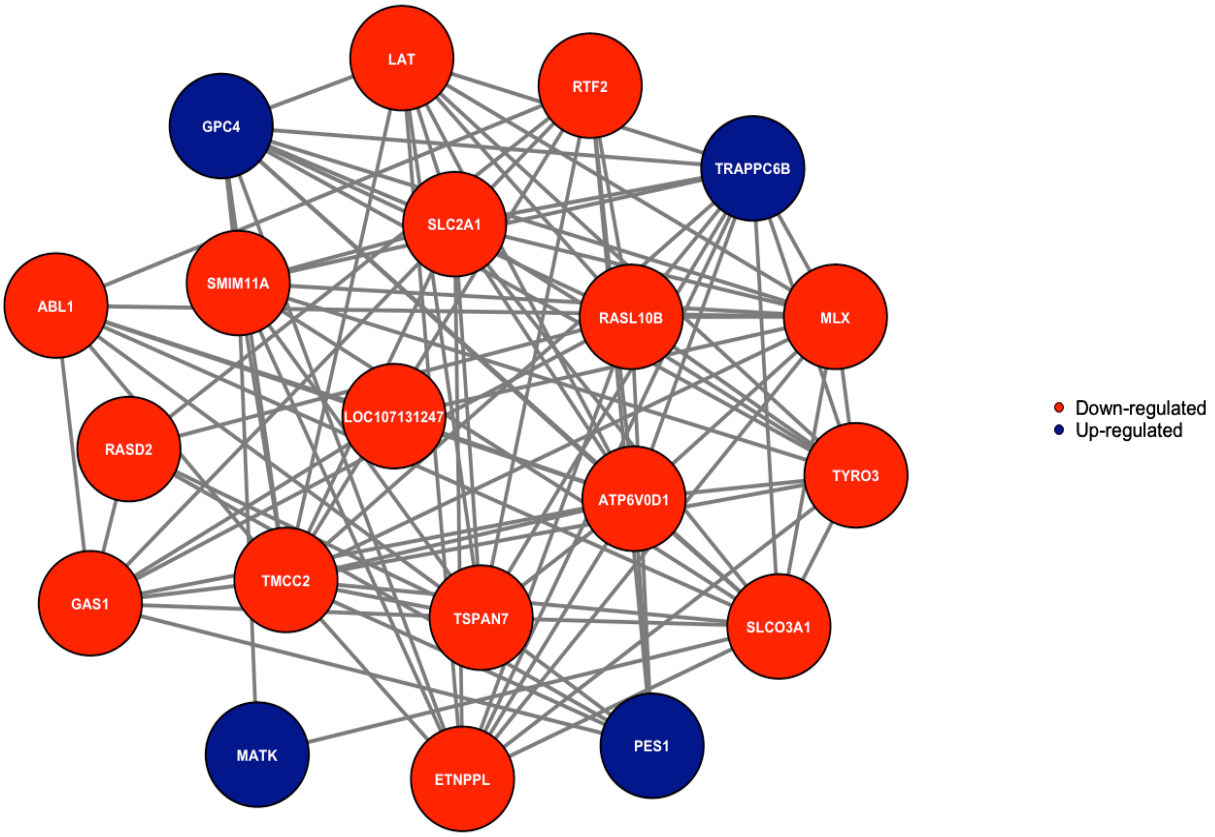

Figure 2.3 *Partial correlation network for top 20 differentially expressed genes (q-value < 0.05) for the effect of diet in muscle tissue. Red and blue nodes represent down- and up-regulated genes in the restricted group (REST).*

The partial correlation network linking the 10 most DEG in muscle with the 10 most DEG in blood is shown in Figure 2.4. On average, each gene was connected to 3.4 genes. The gene *LOC512150* (*MYADM*) showed the highest connectivity (8 genes) between them. Just one gene (*ETNPPL*) was not connected using correlation > |0.50|. *LOC512150*

(*MYADM*) was up-regulated in REST in the blood and *ETNPPL* was down-regulated in the REST in the muscle. In addition, we found 7 DEG simultaneously in muscle and blood: *SPAG17*, *VAT1*, *CABLES1*, *SLC20A2*, *ILF3*, *QDPR*, and *LOC107131247*. Among them, *VAT1*, *CABLES1*, *SLC20A2*, *ILF3*, and *QDPR* were down-regulated in muscle and up-regulated in blood, and *SPAG17* and *LOC107131247* were up-regulated in both.

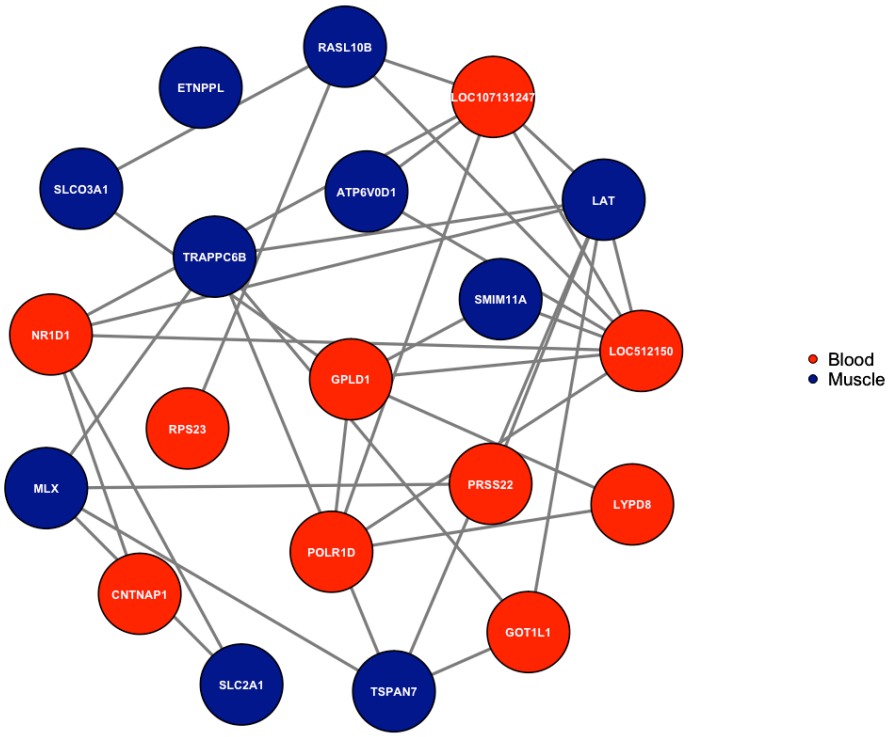

Figure 2.4 *Partial correlation networks for the top 20 differentially expressed genes (DEG; q-value < 0.05) for the effect of diet - 10 from muscle associated with 10 from blood tissue. Red and blue nodes represent differentially expressed genes in blood and muscle tissues, respectively.*

Discussion

Overall Discussion

Alterations in the intra-uterine environment, such as those caused by maternal nutrition during pregnancy, can modify prenatal development, which can lead to positive or negative consequences in the adult life of the animal [2,80]. In addition to the pre-natal vulnerability, the pre-conditioning phase is also critical for offspring development since the animal is going through a stressful period and may have its performance compromised. Therefore, a well-developed immune system, associated with proper development of muscle tissue, would favor the productive performance of the animal. Our study focused on the effect of maternal energy restriction during late gestation on the transcriptomic profile in skeletal muscle and blood tissues in the offspring.

In skeletal muscle tissue, 131 of the 160 DEG were down-regulated in the REST vs CTRL, suggesting that maternal energy restriction during late gestation may limit the expression of genes in the muscle of calves. On the other hand, there was a numerical increase in up-regulation of genes in the REST (410 out of 452) compared to the CTRL group, in the blood of the calves. Although we do not have the data to conclude on this, this clear gene-regulation bias towards one diet or the other could be due to maternal energy restriction causing epigenetic modifications in the fetal genome.

Enrichment Analyses in the Muscle

Enrichment analysis in the muscle showed an overrepresentation of genes associated with metabolism. Most of them are of general categories, such as *regulation of transport* (GO:0051049), *regulation of localization* (GO:0032879), and *regulation of intracellular transport* (GO:0032386), but we also found more relevant categories related to muscle

metabolism such as *positive regulation of actin cytoskeleton reorganization* (GO:2000251)*, positive regulation of collagen biosynthetic process* (GO:0032967), and *positive regulation of collagen metabolic process* (GO:0010714). These functional terms support that maternal nutrition alters metabolic processes in fetal tissues as previously observed by Mortensen et al.[81]. Those authors found that the maternal pre-natal diet with low protein fed at isoenergetic intake in mice caused down-regulation of several pathways related to energetic metabolism processes including glycolysis, citrate cycle, and amino acid degradation, in skeletal muscle in newborns.

DEG for the effect of sex were enriched for very general biological processes, most of which were associated with acid nucleic metabolism (e.g. *histone H3-K27 demethylation* (GO:0071557), *DNA-dependent DNA replication* (GO:0006261), *DNA replication initiation* (GO:0006270), and *DNA repair* (GO:0006281)) in addition to metabolism of carbohydrate and muscle (i.e. *positive regulation of glycoprotein biosynthetic process* (GO:0010560), *regulation of skeletal muscle contraction* (GO:0014819), and *regulation of glycogen metabolic process* (GO:0070873). The enrichment analysis results can be seen on Table 2. These functional terms are related to chromatin biology and epigenetics suggesting that these variations are responsible for the changes in gene expression observed in fetal tissues. Our study supports the hypothesis that males and females have different responses to fetal programming as has been shown previously. Differences in offspring outcomes according to their sex as an effect of fetal programming was reported in sheep [82,83] and cows [84,85].

Major DEG in Muscle Tissue

From the top 10 down-regulated genes in the REST, we identified 5 genes associated with muscle metabolism and development as well as with energy metabolism: (1) *SLCO3A1*,

a membrane transporter of the thyroid hormone involved in satellite cell differentiation [32]; (2) *ATP6V0D1*, involved in oxidative phosphorylation [36]; (3) *SLC2A1*, a facilitator of glucose transport; (4) *GPC4*, involved in cell proliferation [39]; and (5) *RASD2*, expressed in satellite cells and involved in cellular movement and cell cycle. This connection of major genes with energy metabolism associated with the relevant functional terms in the enrichment analysis may indicate a shift in the way the tissues are generating energy in the REST compared with CRTL. We propose that energy is being produced primarily through the glycolytic pathway instead of the oxidative pathway, which is less efficient and occurs as a result of lack of sufficient energy. Concordantly, Daniel et al. [86] showed that maternal dietary restriction during mid-gestation increases the number of fast (type II) muscle fibers, which generate energy through glycolytic pathways, as a compensatory process due to limited energy availability for muscle development. Mitochondrial oxidative phosphorylation is the primary pathway to produce energy for metabolic activities, which generates more adenosine triphosphate (ATP) than glycolysis [87]; however, lack of energy can decrease the number of fast fibers [86], reducing the efficiency of energy production and, consequently, of metabolic activities. In accordance, Byrne et al. [88] suggested that caloric restriction may increase metabolism of amino acids to glucose via gluconeogenesis after finding increased gene expression levels associated with energy metabolism. Moreover, Peñagaricano et al. [9] showed that the maternal source of energy may affect gene expression in the muscle of sheep. These authors observed that dams fed a corn-based diet had greater expression of genes associated with embryonic and fetal development, skeletal muscle, tissue differentiation, muscle myosin complex, and sarcomere organization, than dams fed alfalfa haylage and distillers grains. These results indicate that the energy-restricted diet may be

impairing the development of muscle tissue as well as altering the energy metabolism in the offspring. In addition, Yang et al. [39] reported *GP4* as less expressed due hypermethylation in Chinese pigs, which are known for having less lean muscle mass and low growth rate, suggesting that reduced gene expression may be occurring due epigenetic modifications. The downregulation of *RASD2* in the REST may also indicates that maternal energy restriction alters the proliferation capacity of satellite cells, controlling the skeletal muscle hypertrophy. Raja et al. [41] have shown that energy restriction during gestation alters temporal expression of myogenic regulatory factors in satellite cells impairing the fusion of cells isolated from 3-month old lambs. Therefore, alterations in maternal nutrition during pregnancy may lead to negative effects on postnatal myogenesis.

In contrast, REST had increased expression of genes related to fatty acid and glucose metabolism, such as *ETNPPL*, associated with fatty acid and lipid metabolic process [42]; *SYT3*, involved in glucose metabolism [45]; and *ETFRF1*, involved in fatty acid oxidation [48]. The gene *SYT3* has been shown to be up-regulated in a mutated mice lacking the gene *RORα* and presenting lean phenotype, resistance to high fat diet, and insulin resistance [45]. This finding suggests that the REST presents an impaired glucose metabolism compared with the CTRL group, which may be influencing the increase in expression of genes associated fatty acid metabolism (*ETNPPL* and *ETFRF1).* In addition, the overexpression of *FZD5* in the REST may indicate an disruption on the *Wnt* signaling pathway, which is related to diverse functions in developmental processes, including glucose metabolism [50]. These findings suggest that energy restriction during late pregnancy affects glucose metabolism and enhances fatty acid metabolism.

From the top 10 DEG for the effect of sex, in accordance with the enrichment analyses, we identified genes associated with chromatin biology and epigenetic modifications, such as *KDM6A* and *ICMT* up-regulated in females compared with males. From the top 10 DEG for the effect of sex, in accordance with the enrichment analyses, we identified genes associated with glucose metabolism, such as *GCGR* (up-regulated in males) and *EIF2S3* (up-regulated in females) and chromatin biology and epigenetic modifications, such as *KDM6A* and *ICMT* up-regulation in females compared with males. In accordance, glucose metabolism had been thought to differ between male and female embryos [53]. In addition, Alvarez et al. [89] found higher transcriptional level in females compared with males. We propose that the X-linked genes inactivation in females may occur as a result of an imprinting mechanism leading to a total or partial maternal allele transcriptional repression [89], which can generate an up-regulation of genes in females compared with males (Figure 2.2). These results suggest that the different gene expression levels occur due to difference in sex chromosome dosage.

Enrichment Analysis in the Blood Tissue

For the analysis for DEG in the blood, we focused on the overall effect of diet and its effects as a function of vaccination and weaning. Biological processes related to major immune response activities and response to stress were identified for the effects of vaccination and weaning, respectively, such as *cellular response to DNA damage stimulus* (GO:0006974)*, response to stress* (GO:0006950)*, regulation of lymphocyte activation* (GO:0051249), *response to interleukin-4* (GO:0070670) and *immune system process* (GO:0002376), suggesting that the maternal nutrition during pregnancy influenced the response of the offspring to the immune challenge after weaning and vaccination. It has been

shown that the immune challenge may elicit an acute phase response which decrease feed intake and increases protein demand to support the immune system [12,13] leading to compromised growth. The energy-restricted diet may be impairing the animal`s response in this phase, since the energy metabolism might be compromised and less efficient to synthesize protein for the immune system.

Major DEG in Blood Tissue

Among the top 10 major DEG for the effect of the diet-vs-weaning interaction, we found genes related to immune system and response to stress, such as *KLRK1,* which stimulates natural killer (NK) cells [65] and *INO80D* [73], involved in oxidation-reduction activity. *KLRK1* was down-regulated and *INO80D* was up-regulated in the REST after weaning (Figure 2.5). In accordance with the results in the muscle tissue for the effect of energy-restricted diet, we hypothesized that the REST triggered a more pronounced stress process than the CRTL during the acute response phase.

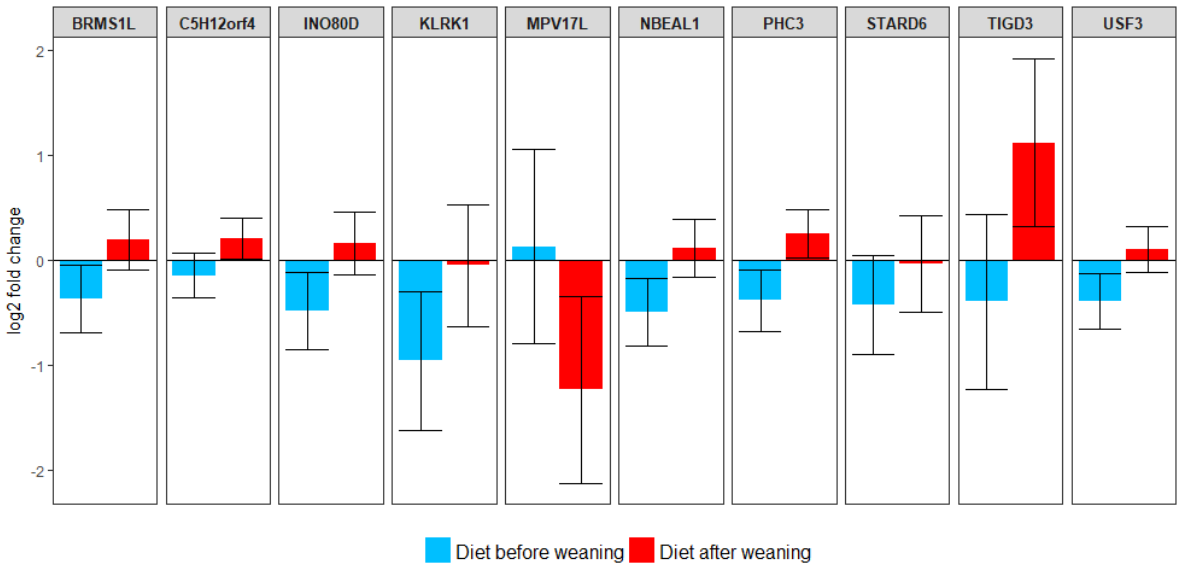

Figure 2.5 *Top 10 differentially expressed genes (q-value < 0.05) showing interaction between the effects of diet and weaning in the blood tissue. Bars represent the log2 fold-change for the effect (P-value < 0.05) of diet before (blue) and after (red) weaning, with error bars representing 95% confidence interval. Positive and negative values represent up- and down-regulation of genes in the restricted group (REST). The name and function of the genes are summarized in Table 5.*

When we looked at the effect of the diet-by-vaccination interaction, we found genes involved in immune response were down-regulated in the REST after vaccination (Figure 2.6), such as *OASL*, which is involved in r*esponse to viral infections* [67]; *KLRF1*, which is expressed on nearly all NK cells and stimulates their cytotoxicity and cytokine release [65]; and *LOC104968634,* an antimicrobial peptide stimulating NK cells cytoxicity. These findings suggest that the CRTL responded better to the immune challenge which may be due to better development of the immune system during pre-natal phase. Also, these data are in agreement with Moriel et al. [18], who used a portion of the data from this study, showed that 70% of energy restriction during the last 40 days of gestation decreased post-weaning vaccination-induced humoral immunity, inflammatory, and physiological stress responses in calves. Our results support the hypothesis that maternal energy restriction during pregnancy can alter gene expression in the offspring associated with immune response which may reflect on the growth performance of the animal.

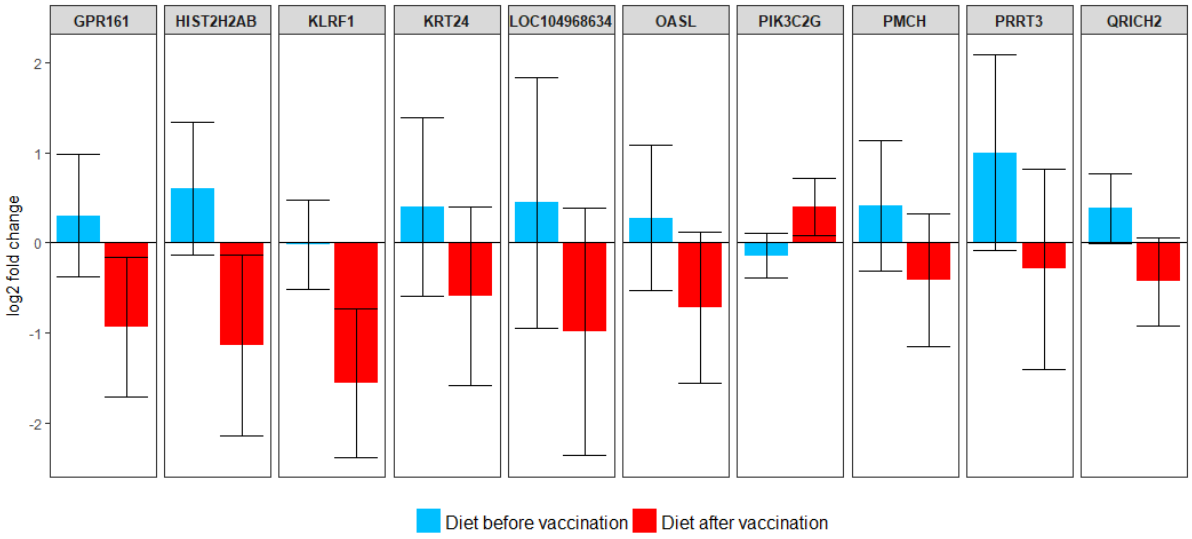

Figure 2.6 *Top 10 differentially expressed genes (q-value < 0.05) showing interaction between the effects of diet and vaccination. Bars represent the log2 fold-change for the effect of diet for before (red) and after (blue) vaccination, and error bars represent 95% confidence interval. Positive and negative values represent up- and down-regulation of genes in the restricted group (REST). The name and function of the genes are summarized on Table 5.*

Gene Networks

From the gene-network for the top 20 major genes in muscle, the genes *ATP6V0D1* and *TMCC2* showed highest connectivity and were negatively correlated to each other. *TMCC2* and *ATP6V0D1* were up- down-regulated in the REST, respectively, and *ATP6V0D1* plays an important role in energy metabolism as discussed previously. All genes showed connectivity with at least 2 other genes.

The result from the partial correlation network linking DEG in muscle and blood (Figure 2.4) showed *LOC512150* (*MYADM*) with highest connectivity (8), and *ETNPPL* with no connectivity. *LOC512150* (*MYADM*) was up-regulated in the REST the blood and *ETNPPL* was down-regulated in the REST in muscle. *LOC512150* (*MYADM*) encodes for cell-surface markers for immune cells, which supports the hypothesis that the CRTL group responded better to the immune challenge compared with the REST.

Among the DEG in both blood and muscle (*SPAG17*, *VAT1*, *CABLES1*, *SLC20A2*, *ILF3*, *QDPR*, and *LOC107131247*), most of them were down-regulated in muscle and up-regulated in blood, except for *SPAG17* and *LOC107131247,* which were down-regulated in both. Among the genes down-regulated in the muscle and up-regulated in blood, some of them are associated with the immune system and stress response, i.e. *ILF3* [90], *QDPR* [91], *CABLES1* [92], and *LOC107131247* [93]. *CABLES1* is a glucocorticoid-activated cell cycle regulator. Glucocorticoids are known to be involved in several growth-related functions, including enhanced response to stress and decreased protein synthesis in skeletal muscle. Therefore, the DEG expressed in both blood and muscle tissue may be playing different roles in each of the tissues.

Conclusion

This study indicates that reducing dietary energy in pregnant cows by 30% during the last 40 days of gestation impacts the transcriptomic profiling of skeletal muscle and blood tissues in the offspring. Maternal energy restriction reduced the expression of genes in the skeletal muscle tissue and increased the gene expression in blood. Specifically, we observed a reduced expression of genes associated with energy metabolism and muscle development in the skeletal muscle tissue. In blood tissue, there was a decrease in expression of genes associated with immune response and stress processes in the REST after weaning and vaccination. Also, we found that DEG in muscle and blood showed a connectivity between them, enhancing the importance of the immune system as a target tissue for the effect of maternal energy restriction during late pregnancy. These finding suggest that energy restriction during late pregnancy may trigger a more pronounced stress response in the offspring that may impair muscle growth and immune development.

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CHAPTER 3. LIMITATIONS AND FUTURE DIRECTIONS

In this thesis, we studied the effect of energy restriction during late gestation in the blood and muscle transcriptome of preconditioned beef calves. Maternal restriction during late gestation was shown to affect the regulation of genes associated with skeletal muscle metabolism and immune system of the offspring.

During the development of this study, some assumptions were considered to overcome limitations. For example, the effect of weaning and vaccination were confounded with the effect of age. We believed this time effect would be minimum due to the small space of time between the collection of samples. To deal with this issue, it would be necessary to replicate the study adding one control group after weaning/vaccination to avoid the age effect. Also, the number of animals was small (6 animal in each group) especially if we think about the high biological variance between samples. Unfortunately, this is a recurrent issue in RNA-seq experiments since the cost of the technology is still a limitation. The number of sires used (two sires) was also a limitation of the study. The use of additional sires would allow us to make more general conclusions. Another limitation in this study was the time-points used for sample collection. For example, collection of muscle and blood samples right after birth would allow us to have a better to understanding of the effects of the intrauterine environment on the post-natal transcriptome profile of the offspring. With an additional time-point, we could compare changes in the expression levels of genes of interest.

This study showed the importance of investigating the blood transcriptome profile as a function of maternal nutrition. Interesting, we identified several DEG associated with the immune response. However, there is little information in the literature regarding the development of the immune system in cattle during prenatal phase. Hence, we believe that in

future studies, the investigation of the blood transcriptome would be beneficial to enhance the current understanding how the immune system of the offspring can be affected by maternal nutrition. In addition, even with small number of samples, we were able to detect between-treatments gene expression variations. It would be also interesting to look the within-treatment variations due to individual differences. If we could perform this type of study at a much larger scale (e.g. 1,000 animals per treatment), we could estimate genetic parameters and perform genomic analyses for several traits as a function of maternal nutrition. The identification of genetic-by-treatment interaction would suggest different families would respond differently depending on the diet provided during gestation. This would have major implications to the livestock industry. Finally, since there is a strong hypothesis that the changes during fetal programming occur due to epigenetic modifications, it would be interesting to look for the DNA methylation and histone acetylation pattern in the offspring. The identification of imprinting genes between the DEG would also be an indicative of epigenetic modification, what would help to support this hypothesis. This study provides a list of candidate genes which have the level of expression affected as an effect of maternal energy restriction during late gestation. Based on the information obtained from the enrichment analysis associated with the most significant DEG, these genes may also be included (i.e. as genomic biomarkers) in future genomic selection methods using functional information.

In conclusion, although several limitations must be recognized, this study was able to show the effect of maternal restriction during late gestation on the gene expression of the offspring.

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APPENDIX. GENE SET ENRICHMENT ANALYSIS

Table 0.1 Gene set enrichment analysis for muscle tissue

GO biological process complete	#genes	FE	*P*-value
Effect of diet			
Positive regulation of transport (GO:0051050)	14	2.92	3.81E-04
Regulation of neuron projection development (GO:0010975)	8	4.27	7.09E-04
Negative regulation of myeloid cell differentiation (GO:0045638)	4	9.56	1.02E-03
Regulation of neuron differentiation (GO:0045664)	9	3.32	1.86E-03
Positive regulation of blood vessel endothelial cell migration (GO:0043536)	3	13.21	1.90E-03
Regulation of transport (GO:0051049)	20	2.08	2.13E-03
Regulation of cell morphogenesis involved in differentiation (GO:0010769)	6	4.55	2.44E-03
Regulation of cell morphogenesis (GO:0022604)	8	3.33	3.28E-03
Regulation of intracellular transport (GO:0032386)	7	3.55	4.16E-03
Regulation of protein targeting (GO:1903533)	4	6.33	4.26E-03
Membrane depolarization (GO:0051899)	3	9.4	4.70E-03
Positive regulation of actin cytoskeleton reorganization (GO:2000251)	2	21.68	4.88E-03
Peptidyl-proline hydroxylation (GO:0019511)	2	21.68	4.88E-03
Regulation of mitochondrion organization (GO:0010821)	5	4.49	5.87E-03
Negative regulation of hemopoiesis (GO:1903707)	4	5.75	5.90E-03
Cell surface receptor signaling pathway (GO:0007166)	20	1.87	6.52E-03
Regulation of nervous system development (GO:0051960)	10	2.55	6.61E-03
Regulation of neurogenesis (GO:0050767)	9	2.68	7.28E-03
Regulation of localization (GO:0032879)	25	1.74	7.58E-03
Regulation of plasma membrane bounded cell projection organization (GO:0120035)	8	2.86	7.79E-03
Positive regulation of collagen biosynthetic process (GO:0032967)	2	16.58	7.80E-03
Positive regulation of collagen metabolic process (GO:0010714)	2	16.58	7.80E-03
Regulation of cell projection organization (GO:0031344)	8	2.83	8.36E-03
Positive regulation of intracellular transport (GO:0032388)	5	4.1	8.45E-03
Regulation of collagen synthetic process (GO:0032965)	2	15.66	8.62E-03
Positive regulation of mitochondrion organization (GO:0010822)	4	5.08	8.95E-03
Regulation of blood vessel endothelial cell migration (GO:0043535)	3	7.29	9.16E-03
Negative regulation of developmental process (GO:0051093)	11	2.3	9.26E-03

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Labyrinthine layer blood vessel development (GO:0060716)	2	14.84	9.49E-03
Positive regulation of GTPase activity (GO:0043547)	7	2.98	1.02E-02
Plasma membrane organization (GO:0007009)	3	6.93	1.04E-02
Positive regulation of protein targeting to mitochondrion (GO:1903955)	3	6.82	1.09E-02
Alpha-amino acid metabolic process (GO:1901605)	5	3.83	1.10E-02
Positive regulation of establishment of protein localization to mitochondrion (GO:1903749)	3	6.71	1.14E-02
Positive regulation of catalytic activity (GO:0043085)	15	2.04	1.18E-02
Cellular response to tumor necrosis factor (GO:0071356)	4	4.66	1.19E-02
Regulation of multicellular organismal metabolic process (GO:0044246)	2	12.81	1.23E-02
Regulation of protein targeting to mitochondrion (GO:1903214)	3	6.41	1.28E-02
Positive regulation of cell communication (GO:0010647)	16	1.89	1.28E-02
Regulation of intracellular protein transport (GO:0033157)	5	3.67	1.30E-02
Endomembrane system organization (GO:0010256)	6	3.17	1.30E-02
Protein hydroxylation (GO:0018126)	2	12.26	1.33E-02
Regulation of cell junction assembly (GO:1901888)	3	6.31	1.33E-02
Positive regulation of cellular component organization (GO:0051130)	13	2.04	1.38E-02
Positive regulation of protein transport (GO:0051222)	7	2.8	1.39E-02
Regulation of vesicle-mediated transport (GO:0060627)	7	2.79	1.41E-02
Pigment cell differentiation (GO:0050931)	2	11.75	1.43E-02
Positive regulation of calcium-mediated signaling (GO:0050850)	2	11.75	1.43E-02
Motor neuron axon guidance (GO:0008045)	2	11.75	1.43E-02
Positive regulation of endothelial cell migration (GO:0010595)	3	6.13	1.44E-02
Regulation of establishment of protein localization to mitochondrion (GO:1903747)	3	6.04	1.49E-02
Positive regulation of signal transduction (GO:0009967)	15	1.92	1.53E-02
Semaphorin-plexin signaling pathway (GO:0071526)	2	11.28	1.54E-02
T cell proliferation (GO:0042098)	2	11.28	1.54E-02
Cellular response to nitrogen compound (GO:1901699)	7	2.73	1.59E-02
Positive regulation of establishment of protein localization (GO:1904951)	7	2.73	1.59E-02
Response to tumor necrosis factor (GO:0034612)	4	4.24	1.62E-02
Regulation of actin cytoskeleton reorganization (GO:2000249)	2	10.84	1.65E-02
Cell-substrate adhesion (GO:0031589)	4	4.15	1.74E-02
Positive regulation of binding (GO:0051099)	4	4.12	1.78E-02

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of secretion by cell (GO:1903530)	8	2.44	1.85E-02
Positive regulation of substrate adhesion-dependent cell spreading (GO:1900026)	2	10.07	1.89E-02
Placenta blood vessel development (GO:0060674)	2	10.07	1.89E-02
Adipose tissue development (GO:0060612)	2	10.07	1.89E-02
Alpha-amino acid catabolic process (GO:1901606)	3	5.49	1.90E-02
Positive regulation of signaling (GO:0023056)	16	1.88	1.94E-02
Positive regulation of hydrolase activity (GO:0051345)	9	2.27	1.97E-02
Plasma membrane bounded cell projection assembly (GO:0120031)	6	2.87	2.01E-02
Positive regulation of intracellular protein transport (GO:0090316)	4	3.94	2.04E-02
Cell projection assembly (GO:0030031)	6	2.85	2.06E-02
Negative regulation of neuron projection development (GO:0010977)	3	5.29	2.09E-02
Regulation of GTPase activity (GO:0043087)	7	2.57	2.11E-02
Positive regulation of ERK1 and ERK2 cascade (GO:0070374)	4	3.89	2.13E-02
Regulation of myeloid cell differentiation (GO:0045637)	4	3.84	2.22E-02
Cellular response to oxygen levels (GO:0071453)	3	5.16	2.22E-02
Maturation of LSU-rRNA (GO:0000470)	2	9.09	2.26E-02
Negative regulation of immune system process (GO:0002683)	6	2.78	2.28E-02
Response to organic cyclic compound (GO:0014070)	8	2.33	2.35E-02
Maturation of 5.8S rRNA (GO:0000460)	2	8.81	2.39E-02
Regulation of sprouting angiogenesis (GO:1903670)	2	8.81	2.39E-02
Cellular amino acid catabolic process (GO:0009063)	3	4.97	2.43E-02
Regulation of cell-matrix adhesion (GO:0001952)	3	4.92	2.51E-02
Regulation of cell aging (GO:0090342)	2	8.54	2.52E-02
Regulation of developmental growth (GO:0048638)	5	3.06	2.56E-02
Small molecule catabolic process (GO:0044282)	5	3.05	2.61E-02
Positive regulation of cell development (GO:0010720)	6	2.69	2.61E-02
Cardiovascular system development (GO:0072358)	7	2.45	2.64E-02
Endothelial cell development (GO:0001885)	2	8.29	2.66E-02
Developmental pigmentation (GO:0048066)	2	8.29	2.66E-02
Negative regulation of myeloid leukocyte differentiation (GO:0002762)	2	8.29	2.66E-02
Second-messenger-mediated signaling (GO:0019932)	4	3.61	2.68E-02
Response to oxygen levels (GO:0070482)	4	3.59	2.73E-02
Positive regulation of cell morphogenesis involved in differentiation (GO:0010770)	3	4.75	2.73E-02
Regulation of secretion (GO:0051046)	8	2.26	2.75E-02
Regulation of lipid storage (GO:0010883)	2	8.05	2.80E-02

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of calcium ion transport into cytosol (GO:0010524)	2	8.05	2.80E-02
Positive regulation of organelle organization (GO:0010638)	8	2.25	2.83E-02
Positive regulation of secretion by cell (GO:1903532)	5	2.96	2.91E-02
Regulation of cellular component organization (GO:0051128)	21	1.6	3.01E-02
Cellular response to organic cyclic compound (GO:0071407)	6	2.59	3.09E-02
Positive regulation of cell-matrix adhesion (GO:0001954)	2	7.42	3.23E-02
Regulation of cell differentiation (GO:0045595)	15	1.75	3.29E-02
Ras protein signal transduction (GO:0007265)	3	4.4	3.29E-02
Regulation of substrate adhesion-dependent cell spreading (GO:1900024)	2	7.23	3.38E-02
Substrate adhesion-dependent cell spreading (GO:0034446)	2	7.23	3.38E-02
Positive regulation of peptide secretion (GO:0002793)	4	3.34	3.42E-02
Positive regulation of epithelial cell migration (GO:0010634)	3	4.31	3.46E-02
Positive regulation of DNA replication (GO:0045740)	2	7.05	3.53E-02
Positive regulation of stress fiber assembly (GO:0051496)	2	7.05	3.53E-02
Labyrinthine layer development (GO:0060711)	2	7.05	3.53E-02
Regulation of lipid localization (GO:1905952)	3	4.23	3.64E-02
Negative regulation of cell projection organization (GO:0031345)	3	4.23	3.64E-02
Positive regulation of intracellular signal transduction (GO:1902533)	10	1.99	3.66E-02
Positive regulation of secretion (GO:0051047)	5	2.77	3.67E-02
Cellular pigmentation (GO:0033059)	2	6.88	3.69E-02
Positive regulation of lipid transport (GO:0032370)	2	6.88	3.69E-02
Organic acid catabolic process (GO:0016054)	4	3.24	3.74E-02
Carboxylic acid catabolic process (GO:0046395)	4	3.24	3.74E-02
Positive regulation of molecular function (GO:0044093)	16	1.74	3.80E-02
Intracellular receptor signaling pathway (GO:0030522)	3	4.15	3.82E-02
Glucose transport (GO:0015758)	2	6.71	3.85E-02
Regulation of cell development (GO:0060284)	9	2.19	3.86E-02
Regulation of peptide transport (GO:0090087)	9	2.19	3.86E-02
I-kappab kinase/NF-kappab signaling (GO:0007249)	2	6.56	4.01E-02
Regulation of ubiquitin-protein transferase activity (GO:0051438)	2	6.56	4.01E-02
Negative regulation of nervous system development (GO:0051961)	4	3.15	4.07E-02
Cellular amino acid metabolic process (GO:0006520)	5	2.69	4.09E-02
Regulation of cell growth (GO:0001558)	5	2.69	4.09E-02

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Response to dsRNA (GO:0043331)	3	4.03	4.10E-02
Negative regulation of BMP signaling pathway (GO:0030514)	2	6.41	4.17E-02
Posttranscriptional gene silencing by RNA (GO:0035194)	2	6.41	4.17E-02
Lymphocyte homeostasis (GO:0002260)	2	6.26	4.34E-02
Peptidyl-tyrosine autophosphorylation (GO:0038083)	2	6.26	4.34E-02
Cellular response to dsRNA (GO:0071359)	2	6.26	4.34E-02
Posttranscriptional gene silencing (GO:0016441)	2	6.26	4.34E-02
Negative regulation of I-kappab kinase/NF-kappab signaling (GO:0043124)	2	6.26	4.34E-02
Hexose transport (GO:0008645)	2	6.26	4.34E-02
Lymphocyte migration (GO:0072676)	2	6.26	4.34E-02
Regulation of growth (GO:0040008)	7	2.19	4.39E-02
Cell proliferation (GO:0008283)	7	2.19	4.39E-02
Regulation of hemopoiesis (GO:1903706)	5	2.63	4.43E-02
Regulation of cell size (GO:0008361)	3	3.88	4.48E-02
Regulation of dendrite morphogenesis (GO:0048814)	2	6.13	4.50E-02
Protein localization to membrane (GO:0072657)	5	2.6	4.61E-02
Positive regulation of neuron differentiation (GO:0045666)	4	3.01	4.64E-02
Positive regulation of MAPK cascade (GO:0043410)	6	2.34	4.64E-02
Monosaccharide transport (GO:0015749)	2	6	4.67E-02
Protein processing (GO:0016485)	3	3.81	4.68E-02
Cell differentiation (GO:0030154)	27	1.45	4.69E-02
Cellular response to molecule of bacterial origin (GO:0071219)	3	3.71	4.99E-02
Effect of sex			
Translation (GO:0006412)	101	5.84	6.58E-42
Peptide biosynthetic process (GO:0043043)	101	5.64	8.81E-41
Amide biosynthetic process (GO:0043604)	103	5.09	3.67E-38
Peptide metabolic process (GO:0006518)	103	4.8	3.40E-36
Cellular amide metabolic process (GO:0043603)	106	4.08	7.70E-32
Cellular macromolecule biosynthetic process (GO:0034645)	181	2.21	4.73E-23
Macromolecule biosynthetic process (GO:0009059)	181	2.17	1.99E-22
Organonitrogen compound biosynthetic process (GO:1901566)	125	2.68	4.22E-22
Cellular nitrogen compound biosynthetic process (GO:0044271)	165	2.11	4.11E-19
Cellular macromolecule metabolic process (GO:0044260)	293	1.62	1.85E-18
Cellular protein metabolic process (GO:0044267)	213	1.84	3.63E-18
Organic substance biosynthetic process (GO:1901576)	203	1.8	2.37E-16
Cellular nitrogen compound metabolic process (GO:0034641)	232	1.7	3.41E-16

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Cellular biosynthetic process (GO:0044249)	198	1.8	9.94E-16
Biosynthetic process (GO:0009058)	204	1.76	1.74E-15
Protein metabolic process (GO:0019538)	232	1.64	1.84E-14
Gene expression (GO:0010467)	168	1.85	3.42E-14
Organelle organization (GO:0006996)	178	1.74	6.55E-13
Macromolecule metabolic process (GO:0043170)	320	1.42	2.33E-12
Cellular component organization or biogenesis (GO:0071840)	252	1.52	2.74E-12
Ribosome biogenesis (GO:0042254)	37	3.65	1.55E-10
Ribosome assembly (GO:0042255)	19	7.13	4.22E-10
Primary metabolic process (GO:0044238)	359	1.32	9.79E-10
Cellular component organization (GO:0016043)	233	1.47	9.91E-10
Nitrogen compound metabolic process (GO:0006807)	335	1.33	1.42E-09
Organonitrogen compound metabolic process (GO:1901564)	249	1.43	2.09E-09
Ribonucleoprotein complex biogenesis (GO:0022613)	44	2.89	2.55E-09
Ribosomal large subunit biogenesis (GO:0042273)	20	5.73	3.88E-09
Cellular metabolic process (GO:0044237)	348	1.3	1.69E-08
Organic substance metabolic process (GO:0071704)	366	1.28	1.79E-08
Regulation of cell cycle (GO:0051726)	66	2.13	3.68E-08
Cellular process (GO:0009987)	588	1.15	6.77E-08
Cell cycle process (GO:0022402)	55	2.26	8.85E-08
Mitotic cell cycle process (GO:1903047)	38	2.74	1.12E-07
Cellular component biogenesis (GO:0044085)	126	1.63	1.30E-07
Cytoskeleton organization (GO:0007010)	67	2.05	1.36E-07
Ribonucleoprotein complex subunit organization (GO:0071826)	28	3.33	1.50E-07
Mitotic cell cycle (GO:0000278)	40	2.57	2.87E-07
Cell cycle (GO:0007049)	64	2.03	3.68E-07
Ribosomal large subunit assembly (GO:0000027)	12	7.44	4.85E-07
Cytoplasmic translation (GO:0002181)	15	5.55	4.85E-07
Ribonucleoprotein complex assembly (GO:0022618)	26	3.27	5.78E-07
Metabolic process (GO:0008152)	378	1.22	1.93E-06
Spindle organization (GO:0007051)	18	4.03	2.31E-06
DNA metabolic process (GO:0006259)	48	2.17	2.34E-06
Cellular response to interleukin-4 (GO:0071353)	8	11.22	3.41E-06
Translational elongation (GO:0006414)	10	7.4	4.53E-06
Response to interleukin-4 (GO:0070670)	8	10.15	6.18E-06
Regulation of cell cycle process (GO:0010564)	39	2.24	8.86E-06
Cellular response to DNA damage stimulus (GO:0006974)	45	2.09	9.40E-06
Chromosome segregation (GO:0007059)	25	2.81	1.08E-05
DNA-dependent DNA replication (GO:0006261)	15	4.08	1.39E-05
Nuclear division (GO:0000280)	25	2.74	1.59E-05

Table A.1 continued

GO biological process complete	#genes	FE	*P*-value
Ribosomal small subunit biogenesis (GO:0042274)	13	4.62	1.62E-05
Mitotic nuclear division (GO:0140014)	17	3.57	1.85E-05
Microtubule-based process (GO:0007017)	42	2.09	2.13E-05
Cellular component assembly (GO:0022607)	106	1.52	2.78E-05
Sister chromatid segregation (GO:0000819)	16	3.61	2.81E-05
DNA replication (GO:0006260)	18	3.26	3.13E-05
Chromosome organization (GO:0051276)	61	1.78	3.13E-05
Biological_process (GO:0008150)	699	1.07	3.31E-05
Positive regulation of biological process (GO:0048518)	219	1.3	3.70E-05
Organelle assembly (GO:0070925)	42	2.01	4.90E-05
DNA repair (GO:0006281)	31	2.28	5.44E-05
Macromolecular complex subunit organization (GO:0043933)	80	1.61	6.09E-05
Regulation of immune system process (GO:0002682)	60	1.74	6.97E-05
Nuclear chromosome segregation (GO:0098813)	20	2.82	7.66E-05
Positive regulation of cellular process (GO:0048522)	198	1.3	8.62E-05
Mitotic spindle organization (GO:0007052)	11	4.51	8.68E-05
Organelle fission (GO:0048285)	25	2.49	8.89E-05
Regulation of mitotic cell cycle (GO:0007346)	34	2.12	1.14E-04
Mitotic sister chromatid segregation (GO:0000070)	13	3.73	1.18E-04
Regulation of small GTPase mediated signal transduction (GO:0051056)	22	2.56	1.26E-04
Response to stress (GO:0006950)	124	1.41	1.56E-04
Regulation of lymphocyte activation (GO:0051249)	27	2.3	1.69E-04
rRNA metabolic process (GO:0016072)	19	2.72	1.74E-04
Microtubule cytoskeleton organization involved in mitosis (GO:1902850)	12	3.76	1.96E-04
Microtubule cytoskeleton organization (GO:0000226)	30	2.16	1.98E-04

#genes: number of genes associated with the GO term in the list of differentially expressed genes (DEG, q-value < 0.05)
FE: Fold Enrichment

Table 0.2 Gene set enrichment analysis for blood tissue

GO biological process complete	#genes	FE	*P*-value
Effect of weaning			
Translation (GO:0006412)	101	5.84	6.58E-42
Peptide biosynthetic process (GO:0043043)	101	5.64	8.81E-41
Amide biosynthetic process (GO:0043604)	103	5.09	3.67E-38
Peptide metabolic process (GO:0006518)	103	4.8	3.40E-36
Cellular amide metabolic process (GO:0043603)	106	4.08	7.70E-32
Cellular macromolecule biosynthetic process (GO:0034645)	181	2.21	4.73E-23
Macromolecule biosynthetic process (GO:0009059)	181	2.17	1.99E-22
Organonitrogen compound biosynthetic process (GO:1901566)	125	2.68	4.22E-22
Cellular nitrogen compound biosynthetic process (GO:0044271)	165	2.11	4.11E-19
Cellular macromolecule metabolic process (GO:0044260)	293	1.62	1.85E-18
Cellular protein metabolic process (GO:0044267)	213	1.84	3.63E-18
Organic substance biosynthetic process (GO:1901576)	203	1.8	2.37E-16
Cellular nitrogen compound metabolic process (GO:0034641)	232	1.7	3.41E-16
Cellular biosynthetic process (GO:0044249)	198	1.8	9.94E-16
Biosynthetic process (GO:0009058)	204	1.76	1.74E-15
Protein metabolic process (GO:0019538)	232	1.64	1.84E-14
Gene expression (GO:0010467)	168	1.85	3.42E-14
Organelle organization (GO:0006996)	178	1.74	6.55E-13
Macromolecule metabolic process (GO:0043170)	320	1.42	2.33E-12
Cellular component organization or biogenesis (GO:0071840)	252	1.52	2.74E-12
Ribosome biogenesis (GO:0042254)	37	3.65	1.55E-10
Ribosome assembly (GO:0042255)	19	7.13	4.22E-10
Primary metabolic process (GO:0044238)	359	1.32	9.79E-10
Cellular component organization (GO:0016043)	233	1.47	9.91E-10
Nitrogen compound metabolic process (GO:0006807)	335	1.33	1.42E-09
Organonitrogen compound metabolic process (GO:1901564)	249	1.43	2.09E-09
Ribonucleoprotein complex biogenesis (GO:0022613)	44	2.89	2.55E-09
Ribosomal large subunit biogenesis (GO:0042273)	20	5.73	3.88E-09
Cellular metabolic process (GO:0044237)	348	1.3	1.69E-08
Organic substance metabolic process (GO:0071704)	366	1.28	1.79E-08
Regulation of cell cycle (GO:0051726)	66	2.13	3.68E-08
Cellular process (GO:0009987)	588	1.15	6.77E-08
Cell cycle process (GO:0022402)	55	2.26	8.85E-08
Mitotic cell cycle process (GO:1903047)	38	2.74	1.12E-07

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Cellular component biogenesis (GO:0044085)	126	1.63	1.30E-07
Cytoskeleton organization (GO:0007010)	67	2.05	1.36E-07
Ribonucleoprotein complex subunit organization (GO:0071826)	28	3.33	1.50E-07
Mitotic cell cycle (GO:0000278)	40	2.57	2.87E-07
Cell cycle (GO:0007049)	64	2.03	3.68E-07
Ribosomal large subunit assembly (GO:0000027)	12	7.44	4.85E-07
Cytoplasmic translation (GO:0002181)	15	5.55	4.85E-07
Ribonucleoprotein complex assembly (GO:0022618)	26	3.27	5.78E-07
Metabolic process (GO:0008152)	378	1.22	1.93E-06
Spindle organization (GO:0007051)	18	4.03	2.31E-06
DNA metabolic process (GO:0006259)	48	2.17	2.34E-06
Cellular response to interleukin-4 (GO:0071353)	8	11.22	3.41E-06
Translational elongation (GO:0006414)	10	7.4	4.53E-06
Response to interleukin-4 (GO:0070670)	8	10.15	6.18E-06
Regulation of cell cycle process (GO:0010564)	39	2.24	8.86E-06
Cellular response to DNA damage stimulus (GO:0006974)	45	2.09	9.40E-06
Chromosome segregation (GO:0007059)	25	2.81	1.08E-05
DNA-dependent DNA replication (GO:0006261)	15	4.08	1.39E-05
Nuclear division (GO:0000280)	25	2.74	1.59E-05
Ribosomal small subunit biogenesis (GO:0042274)	13	4.62	1.62E-05
Mitotic nuclear division (GO:0140014)	17	3.57	1.85E-05
Microtubule-based process (GO:0007017)	42	2.09	2.13E-05
Cellular component assembly (GO:0022607)	106	1.52	2.78E-05
Sister chromatid segregation (GO:0000819)	16	3.61	2.81E-05
DNA replication (GO:0006260)	18	3.26	3.13E-05
Chromosome organization (GO:0051276)	61	1.78	3.13E-05
Biological_process (GO:0008150)	699	1.07	3.31E-05
Positive regulation of biological process (GO:0048518)	219	1.3	3.70E-05
Organelle assembly (GO:0070925)	42	2.01	4.90E-05
DNA repair (GO:0006281)	31	2.28	5.44E-05
Macromolecular complex subunit organization (GO:0043933)	80	1.61	6.09E-05
Regulation of immune system process (GO:0002682)	60	1.74	6.97E-05
Nuclear chromosome segregation (GO:0098813)	20	2.82	7.66E-05
Positive regulation of cellular process (GO:0048522)	198	1.3	8.62E-05
Mitotic spindle organization (GO:0007052)	11	4.51	8.68E-05
Organelle fission (GO:0048285)	25	2.49	8.89E-05
Regulation of mitotic cell cycle (GO:0007346)	34	2.12	1.14E-04
Mitotic sister chromatid segregation (GO:0000070)	13	3.73	1.18E-04
Regulation of small GTPase mediated signal transduction (GO:0051056)	22	2.56	1.26E-04

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Response to stress (GO:0006950)	124	1.41	1.56E-04
Regulation of lymphocyte activation (GO:0051249)	27	2.3	1.69E-04
rRNA metabolic process (GO:0016072)	19	2.72	1.74E-04
Microtubule cytoskeleton organization involved in mitosis (GO:1902850)	12	3.76	1.96E-04
Microtubule cytoskeleton organization (GO:0000226)	30	2.16	1.98E-04
Positive regulation of response to stimulus (GO:0048584)	86	1.52	2.07E-04
Macromolecular complex disassembly (GO:0032984)	12	3.55	3.16E-04
Positive regulation of response to stimulus (GO:0048584)	86	1.52	2.07E-04
Macromolecular complex disassembly (GO:0032984)	12	3.55	3.16E-04
Effect of vaccination			
Localization (GO:0051179)	141	1.61	3.46E-09
Establishment of localization (GO:0051234)	110	1.6	6.83E-07
Immune system process (GO:0002376)	58	1.93	2.65E-06
Transport (GO:0006810)	104	1.57	3.50E-06
Positive regulation of gtpase activity (GO:0043547)	22	3.18	4.20E-06
Cellular response to chemical stimulus (GO:0070887)	67	1.75	1.25E-05
Regulation of gtpase activity (GO:0043087)	23	2.87	1.27E-05
Regulation of response to stimulus (GO:0048583)	97	1.54	2.14E-05
Positive regulation of response to stimulus (GO:0048584)	56	1.78	3.82E-05
Positive regulation of hydrolase activity (GO:0051345)	28	2.4	3.93E-05
Cellular response to organic substance (GO:0071310)	55	1.77	5.19E-05
Cell differentiation (GO:0030154)	84	1.53	8.59E-05
Regulation of response to external stimulus (GO:0032101)	26	2.35	9.76E-05
Transition metal ion transport (GO:0000041)	9	5.07	1.32E-04
Cellular homeostasis (GO:0019725)	28	2.24	1.36E-04
Developmental process (GO:0032502)	118	1.39	1.65E-04
Regulation of hydrolase activity (GO:0051336)	40	1.92	1.71E-04
Myeloid leukocyte activation (GO:0002274)	8	5.47	1.93E-04
Positive regulation of response to external stimulus (GO:0032103)	14	3.22	2.06E-04
Regulation of signaling (GO:0023051)	83	1.5	2.22E-04
Regulation of signal transduction (GO:0009966)	77	1.53	2.22E-04
Regulation of localization (GO:0032879)	67	1.59	2.35E-04
Cellular developmental process (GO:0048869)	84	1.49	2.42E-04
Copper ion transport (GO:0006825)	4	15.97	2.58E-04
Substrate adhesion-dependent cell spreading (GO:0034446)	6	7.37	2.93E-04
Iron ion transmembrane transport (GO:0034755)	4	14.74	3.32E-04

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of catalytic activity (GO:0043085)	40	1.85	3.47E-04
Transmembrane transport (GO:0055085)	42	1.81	3.57E-04
Regulation of actin cytoskeleton reorganization (GO:2000249)	5	9.21	3.83E-04
Regulation of immune system process (GO:0002682)	36	1.88	3.97E-04
Regulation of response to stress (GO:0080134)	36	1.88	4.02E-04
Retina vasculature development in camera-type eye (GO:0061298)	4	13.69	4.21E-04
Regulation of catalytic activity (GO:0050790)	61	1.59	4.75E-04
Tissue remodeling (GO:0048771)	8	4.73	4.78E-04
Regulation of cell communication (GO:0010646)	81	1.48	4.78E-04
Leukocyte migration (GO:0050900)	11	3.51	4.95E-04
Iron ion transport (GO:0006826)	6	6.53	5.25E-04
Regulation of endocytosis (GO:0030100)	11	3.47	5.49E-04
Regulation of cell motility (GO:2000145)	27	2.09	5.50E-04
Cellular response to oxygen-containing compound (GO:1901701)	25	2.12	5.89E-04
Response to wounding (GO:0009611)	15	2.75	6.04E-04
Positive regulation of molecular function (GO:0044093)	46	1.7	6.32E-04
Myeloid leukocyte differentiation (GO:0002573)	8	4.51	6.42E-04
Homeostatic process (GO:0042592)	42	1.76	6.55E-04
Regulation of molecular function (GO:0065009)	84	1.45	6.60E-04
Regulation of cell migration (GO:0030334)	26	2.11	6.89E-04
Response to stress (GO:0006950)	73	1.49	7.64E-04
Ion transport (GO:0006811)	43	1.73	8.25E-04
Cation transport (GO:0006812)	31	1.91	8.38E-04
Metal ion transport (GO:0030001)	24	2.09	8.82E-04
Cell activation (GO:0001775)	21	2.22	8.82E-04
Cell migration (GO:0016477)	26	2.04	9.03E-04
Plasma membrane repair (GO:0001778)	3	20.53	9.16E-04
Cellular response to macrophage colony-stimulating factor stimulus (GO:0036006)	3	20.53	9.16E-04
Response to macrophage colony-stimulating factor (GO:0036005)	3	20.53	9.16E-04
Transition metal ion homeostasis (GO:0055076)	9	3.82	9.19E-04
Regulation of cellular component movement (GO:0051270)	28	1.99	9.28E-04
Inflammatory response (GO:0006954)	16	2.52	9.83E-04
Regulation of biological quality (GO:0065008)	85	1.42	1.00E-03
Wound healing (GO:0042060)	13	2.83	1.08E-03
Negative regulation of alpha-beta T cell activation (GO:0046636)	4	10.08	1.12E-03

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of signal transduction (GO:0009967)	40	1.74	1.13E-03
Cellular chemical homeostasis (GO:0055082)	22	2.17	1.14E-03
Cellular response to iron ion (GO:0071281)	3	17.96	1.24E-03
Iron ion import across plasma membrane (GO:0098711)	3	17.96	1.24E-03
T cell chemotaxis (GO:0010818)	3	17.96	1.24E-03
Multicellular organismal iron ion homeostasis (GO:0060586)	3	17.96	1.24E-03
Positive regulation of signaling (GO:0023056)	42	1.68	1.29E-03
Leukocyte activation (GO:0045321)	18	2.36	1.36E-03
Defense response (GO:0006952)	32	1.83	1.37E-03
Chemical homeostasis (GO:0048878)	29	1.93	1.37E-03
Regulated exocytosis (GO:0045055)	8	3.95	1.42E-03
Inorganic cation transmembrane transport (GO:0098662)	22	2.08	1.62E-03
Positive regulation of endoplasmic reticulum unfolded protein response (GO:1900103)	3	15.97	1.63E-03
Response to organic substance (GO:0010033)	59	1.52	1.69E-03
Regulation of locomotion (GO:0040012)	27	1.91	1.70E-03
Response to cytokine (GO:0034097)	26	1.97	1.75E-03
Regulation of intracellular signal transduction (GO:1902531)	46	1.61	1.84E-03
Leukocyte differentiation (GO:0002521)	15	2.43	1.99E-03
Endomembrane system organization (GO:0010256)	14	2.51	2.06E-03
Cation transmembrane transport (GO:0098655)	24	1.96	2.17E-03
Regulation of vesicle-mediated transport (GO:0060627)	17	2.31	2.18E-03
Positive regulation of cellular component movement (GO:0051272)	17	2.3	2.22E-03
Small GTPase mediated signal transduction (GO:0007264)	12	2.72	2.24E-03
Locomotion (GO:0040011)	32	1.78	2.32E-03
Positive regulation of cellular process (GO:0048522)	111	1.31	2.35E-03
Organic substance transport (GO:0071702)	50	1.55	2.37E-03
Immune response (GO:0006955)	30	1.8	2.42E-03
Divalent metal ion export (GO:0070839)	2	47.9	2.44E-03
Positive regulation of lysosomal protein catabolic process (GO:1905167)	2	47.9	2.44E-03
Immune system development (GO:0002520)	23	1.99	2.45E-03
Positive regulation of cell communication (GO:0010647)	41	1.65	2.46E-03
Plasma membrane organization (GO:0007009)	6	4.71	2.46E-03
Cellular copper ion homeostasis (GO:0006878)	3	13.06	2.61E-03

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Iron ion import (GO:0097286)	3	13.06	2.61E-03
Response to tumor necrosis factor (GO:0034612)	9	3.24	2.64E-03
Divalent metal ion transport (GO:0070838)	13	2.53	2.75E-03
Anatomical structure development (GO:0048856)	105	1.32	2.77E-03
Cellular transition metal ion homeostasis (GO:0046916)	7	3.94	2.81E-03
Divalent inorganic cation transport (GO:0072511)	13	2.52	2.85E-03
Organic anion transport (GO:0015711)	17	2.22	2.88E-03
Cellular response to cytokine stimulus (GO:0071345)	23	1.95	2.92E-03
Intracellular pH reduction (GO:0051452)	4	7.37	3.10E-03
Carbohydrate derivative catabolic process (GO:1901136)	8	3.45	3.13E-03
Negative regulation of leukocyte activation (GO:0002695)	8	3.45	3.13E-03
Hematopoietic or lymphoid organ development (GO:0048534)	22	1.99	3.17E-03
Regulation of cholesterol homeostasis (GO:2000188)	3	11.98	3.22E-03
Negative regulation of type 2 immune response (GO:0002829)	3	11.98	3.22E-03
Positive regulation of protein metabolic process (GO:0051247)	42	1.6	3.31E-03
Response to oxygen-containing compound (GO:1901700)	31	1.74	3.34E-03
Inorganic ion transmembrane transport (GO:0098660)	24	1.94	3.35E-03
Vesicle fusion (GO:0006906)	7	3.81	3.37E-03
Cellular monovalent inorganic cation homeostasis (GO:0030004)	7	3.81	3.37E-03
Positive regulation of biological process (GO:0048518)	120	1.28	3.49E-03
Negative regulation of leukocyte mediated immunity (GO:0002704)	4	7.1	3.51E-03
Organelle membrane fusion (GO:0090174)	7	3.77	3.57E-03
Animal organ development (GO:0048513)	66	1.43	3.71E-03
Regulation of MAPK cascade (GO:0043408)	22	1.94	3.82E-03
Myeloid cell differentiation (GO:0030099)	10	2.83	3.90E-03
Cellular ion homeostasis (GO:0006873)	19	2.06	3.95E-03
Inorganic ion homeostasis (GO:0098771)	21	2.02	3.97E-03
Ion transmembrane transport (GO:0034220)	30	1.76	3.98E-03
Purine nucleobase transport (GO:0006863)	2	31.93	4.01E-03
Wnt signaling pathway, calcium modulating pathway (GO:0007223)	2	31.93	4.01E-03
Purine nucleobase transmembrane transport (GO:1904823)	2	31.93	4.01E-03

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of amyloid-beta clearance (GO:1900223)	2	31.93	4.01E-03
Copper ion transmembrane transport (GO:0035434)	2	31.93	4.01E-03
Pyrimidine nucleobase transport (GO:0015855)	2	31.93	4.01E-03
Nucleobase transport (GO:0015851)	2	31.93	4.01E-03
Positive regulation of protein catabolic process in the vacuole (GO:1904352)	2	31.93	4.01E-03
Response to muscle activity involved in regulation of muscle adaptation (GO:0014873)	2	31.93	4.01E-03
Response to interferon-gamma (GO:0034341)	6	4.23	4.06E-03
Positive regulation of cell motility (GO:2000147)	16	2.2	4.11E-03
Regulation of small GTPase mediated signal transduction (GO:0051056)	12	2.51	4.20E-03
Regulation of leukocyte chemotaxis (GO:0002688)	7	3.64	4.23E-03
Ph reduction (GO:0045851)	4	6.61	4.42E-03
Import into cell (GO:0098657)	18	2.12	4.51E-03
Positive regulation of ERK1 and ERK2 cascade (GO:0070374)	9	2.97	4.51E-03
Regulation of Ras protein signal transduction (GO:0046578)	11	2.61	4.59E-03
Copper ion homeostasis (GO:0055070)	3	10.26	4.67E-03
Localization of cell (GO:0051674)	26	1.79	4.74E-03
Cell motility (GO:0048870)	26	1.79	4.74E-03
Ion homeostasis (GO:0050801)	21	1.94	4.89E-03
Membrane organization (GO:0061024)	21	1.94	4.89E-03
Membrane fusion (GO:0061025)	9	2.93	4.91E-03
Drug transport (GO:0015893)	8	3.17	5.10E-03
Cell chemotaxis (GO:0060326)	9	2.91	5.11E-03
Regulation of ERK1 and ERK2 cascade (GO:0070372)	11	2.56	5.26E-03
System development (GO:0048731)	86	1.34	5.29E-03
Biological regulation (GO:0065007)	262	1.13	5.35E-03
Myeloid dendritic cell differentiation (GO:0043011)	3	9.58	5.52E-03
T cell migration (GO:0072678)	3	9.58	5.52E-03
Anion transport (GO:0006820)	20	2	5.57E-03
Cation homeostasis (GO:0055080)	20	1.97	5.87E-03
Hemopoiesis (GO:0030097)	20	1.97	5.87E-03
Glycerol-3-phosphate biosynthetic process (GO:0046167)	2	23.95	5.93E-03
Positive regulation of PERK-mediated unfolded protein response (GO:1903899)	2	23.95	5.93E-03
Ferrous iron transmembrane transport (GO:1903874)	2	23.95	5.93E-03
Ferrous iron transport (GO:0015684)	2	23.95	5.93E-03
Cellular cation homeostasis (GO:0030003)	18	2.01	6.04E-03

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Osteoclast differentiation (GO:0030316)	4	5.99	6.06E-03
Negative regulation of cellular process (GO:0048523)	98	1.31	6.10E-03
Positive regulation of immune system process (GO:0002684)	23	1.83	6.18E-03
Negative regulation of cell activation (GO:0050866)	8	3.04	6.39E-03
Response to iron ion (GO:0010039)	3	8.98	6.45E-03
Regulation of lymphocyte chemotaxis (GO:1901623)	3	8.98	6.45E-03
Regulation of endoplasmic reticulum unfolded protein response (GO:1900101)	3	8.98	6.45E-03
Multicellular organism development (GO:0007275)	94	1.31	6.47E-03
Activation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0006919)	5	4.52	6.59E-03
Positive regulation of cell migration (GO:0030335)	15	2.14	6.63E-03
Synaptic vesicle cycle (GO:0099504)	6	3.78	6.70E-03
Positive regulation of inflammatory response (GO:0050729)	6	3.78	6.70E-03
Receptor-mediated endocytosis (GO:0006898)	9	2.78	6.76E-03
Lysosome organization (GO:0007040)	5	4.44	7.08E-03
Lytic vacuole organization (GO:0080171)	5	4.44	7.08E-03
Leukocyte cell-cell adhesion (GO:0007159)	4	5.64	7.35E-03
Non-canonical Wnt signaling pathway (GO:0035567)	4	5.64	7.35E-03
Myeloid dendritic cell activation (GO:0001773)	3	8.45	7.48E-03
Negative regulation of cell junction assembly (GO:1901889)	3	8.45	7.48E-03
Myeloid leukocyte migration (GO:0097529)	6	3.68	7.52E-03
Regulation of alpha-beta T cell activation (GO:0046634)	5	4.35	7.60E-03
Forebrain generation of neurons (GO:0021872)	5	4.35	7.60E-03
Secretion by cell (GO:0032940)	14	2.19	7.78E-03
Regulation of cellular response to stress (GO:0080135)	19	1.97	7.85E-03
Regulation of cellular pH (GO:0030641)	6	3.64	7.95E-03
Response to stimulus (GO:0050896)	170	1.19	8.06E-03
Mo-molybdopterin cofactor biosynthetic process (GO:0006777)	2	19.16	8.19E-03
Virion attachment to host cell (GO:0019062)	2	19.16	8.19E-03
Hard palate development (GO:0060022)	2	19.16	8.19E-03
Adhesion of symbiont to host cell (GO:0044650)	2	19.16	8.19E-03
Regulation of lysosomal protein catabolic process (GO:1905165)	2	19.16	8.19E-03
Negative regulation of macrophage activation (GO:0043031)	2	19.16	8.19E-03
Copper ion import (GO:0015677)	2	19.16	8.19E-03
Neutrophil clearance (GO:0097350)	2	19.16	8.19E-03

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Amyloid-beta clearance (GO:0097242)	2	19.16	8.19E-03
Mo-molybdopterin cofactor metabolic process (GO:0019720)	2	19.16	8.19E-03
Dichotomous subdivision of terminal units involved in salivary gland branching (GO:0060666)	2	19.16	8.19E-03
Inactivation of MAPK activity (GO:0000188)	3	7.98	8.59E-03
Macrophage activation (GO:0042116)	3	7.98	8.59E-03
Endocytosis (GO:0006897)	16	2.1	8.79E-03
Bone remodeling (GO:0046849)	4	5.32	8.82E-03
Iron ion homeostasis (GO:0055072)	6	3.55	8.88E-03
Cellular response to lipid (GO:0071396)	14	2.14	8.90E-03
Monovalent inorganic cation homeostasis (GO:0055067)	7	3.13	9.03E-03
Positive regulation of cellular protein metabolic process (GO:0032270)	38	1.54	9.14E-03
Positive regulation of locomotion (GO:0040017)	16	2.06	9.41E-03
Organelle fusion (GO:0048284)	7	3.1	9.45E-03
Exocytosis (GO:0006887)	9	2.61	9.78E-03
Wnt signaling pathway, planar cell polarity pathway (GO:0060071)	3	7.56	9.80E-03
Acetylcholine receptor signaling pathway (GO:0095500)	3	7.56	9.80E-03
Positive regulation of antigen receptor-mediated signaling pathway (GO:0050857)	3	7.56	9.80E-03
Cellular response to acetylcholine (GO:1905145)	3	7.56	9.80E-03
Response to acetylcholine (GO:1905144)	3	7.56	9.80E-03
Signal transduction involved in cellular response to ammonium ion (GO:1903831)	3	7.56	9.80E-03
Negative regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (GO:0002823)	3	7.56	9.80E-03
Regulation of antigen receptor-mediated signaling pathway (GO:0050854)	4	5.04	1.05E-02
Lipid transport (GO:0006869)	11	2.31	1.05E-02
Vesicle organization (GO:0016050)	11	2.31	1.05E-02
Cellular defense response (GO:0006968)	2	15.97	1.08E-02
Negative regulation of endothelial cell differentiation (GO:0045602)	2	15.97	1.08E-02
Adhesion of symbiont to host (GO:0044406)	2	15.97	1.08E-02
Prosthetic group metabolic process (GO:0051189)	2	15.97	1.08E-02
Molybdopterin cofactor metabolic process (GO:0043545)	2	15.97	1.08E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of skeletal muscle tissue regeneration (GO:0043416)	2	15.97	1.08E-02
Positive regulation of skeletal muscle tissue regeneration (GO:0043415)	2	15.97	1.08E-02
Regulation of protein catabolic process in the vacuole (GO:1904350)	2	15.97	1.08E-02
Kidney vasculature morphogenesis (GO:0061439)	2	15.97	1.08E-02
Renal system vasculature morphogenesis (GO:0061438)	2	15.97	1.08E-02
Negative regulation of T cell cytokine production (GO:0002725)	2	15.97	1.08E-02
Vacuolar acidification (GO:0007035)	3	7.19	1.11E-02
Negative regulation of cytokine production involved in immune response (GO:0002719)	3	7.19	1.11E-02
Negative regulation of lymphocyte mediated immunity (GO:0002707)	3	7.19	1.11E-02
Establishment of organelle localization (GO:0051656)	13	2.16	1.14E-02
Secretion (GO:0046903)	16	1.97	1.17E-02
Calcium ion regulated exocytosis (GO:0017156)	5	3.86	1.20E-02
Granulocyte migration (GO:0097530)	5	3.86	1.20E-02
Positive regulation of endocytosis (GO:0045807)	6	3.3	1.21E-02
Positive regulation of defense response (GO:0031349)	10	2.37	1.22E-02
Purine-containing compound catabolic process (GO:0072523)	4	4.79	1.23E-02
Regulation of pH (GO:0006885)	6	3.27	1.27E-02
Positive regulation of response to wounding (GO:1903036)	4	4.67	1.33E-02
Positive regulation of protein kinase B signaling (GO:0051897)	5	3.74	1.35E-02
Regulation of leukocyte migration (GO:0002685)	8	2.64	1.36E-02
Regulation of melanocyte differentiation (GO:0045634)	2	13.69	1.37E-02
Neutrophil activation involved in immune response (GO:0002283)	2	13.69	1.37E-02
Purine nucleobase catabolic process (GO:0006145)	2	13.69	1.37E-02
Arginine transport (GO:0015809)	2	13.69	1.37E-02
Regulation of PERK-mediated unfolded protein response (GO:1903897)	2	13.69	1.37E-02
Response to stimulus involved in regulation of muscle adaptation (GO:0014874)	2	13.69	1.37E-02
Lipid localization (GO:0010876)	12	2.23	1.37E-02
Negative regulation of biological process (GO:0048519)	103	1.26	1.39E-02
Regulation of type 2 immune response (GO:0002828)	3	6.53	1.40E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Negative regulation of adaptive immune response (GO:0002820)	3	6.53	1.40E-02
Forebrain neuron development (GO:0021884)	3	6.53	1.40E-02
Regulation of granulocyte chemotaxis (GO:0071622)	4	4.56	1.43E-02
Cell activation involved in immune response (GO:0002263)	8	2.61	1.46E-02
Regulation of chemotaxis (GO:0050920)	8	2.61	1.46E-02
Negative regulation of cellular component movement (GO:0051271)	10	2.3	1.46E-02
Positive regulation of MAPK cascade (GO:0043410)	15	1.98	1.48E-02
Intracellular signal transduction (GO:0035556)	37	1.52	1.54E-02
Forebrain neuron differentiation (GO:0021879)	4	4.46	1.54E-02
Regulation of inflammatory response (GO:0050727)	10	2.28	1.55E-02
Regulation of cell junction assembly (GO:1901888)	5	3.57	1.60E-02
Cellular response to hypoxia (GO:0071456)	5	3.57	1.60E-02
Regulation of multicellular organismal process (GO:0051239)	60	1.37	1.61E-02
Vesicle-mediated transport in synapse (GO:0099003)	6	3.09	1.61E-02
Cellular response to tumor necrosis factor (GO:0071356)	7	2.77	1.63E-02
Fat cell differentiation (GO:0045444)	6	3.06	1.68E-02
Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (GO:0002822)	6	3.06	1.68E-02
Leukotriene biosynthetic process (GO:0019370)	2	11.98	1.68E-02
Glycerol-3-phosphate metabolic process (GO:0006072)	2	11.98	1.68E-02
Regulation of pigment cell differentiation (GO:0050932)	2	11.98	1.68E-02
Regulation of amyloid-beta clearance (GO:1900221)	2	11.98	1.68E-02
Neuron remodeling (GO:0016322)	2	11.98	1.68E-02
Granzyme-mediated apoptotic signaling pathway (GO:0008626)	2	11.98	1.68E-02
Retina vasculature morphogenesis in camera-type eye (GO:0061299)	2	11.98	1.68E-02
Type 2 immune response (GO:0042092)	2	11.98	1.68E-02
Dichotomous subdivision of an epithelial terminal unit (GO:0060600)	2	11.98	1.68E-02
T-helper 17 cell differentiation (GO:0072539)	2	11.98	1.68E-02
T-helper 17 type immune response (GO:0072538)	2	11.98	1.68E-02
Purine-containing compound transmembrane transport (GO:0072530)	2	11.98	1.68E-02
Icosanoid metabolic process (GO:0006690)	5	3.52	1.69E-02
Phospholipid transport (GO:0015914)	5	3.52	1.69E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of endothelial cell differentiation (GO:0045601)	3	5.99	1.73E-02
T cell mediated immunity (GO:0002456)	3	5.99	1.73E-02
Cellular response to vascular endothelial growth factor stimulus (GO:0035924)	3	5.99	1.73E-02
Regulation of macrophage activation (GO:0043030)	3	5.99	1.73E-02
Endoplasmic reticulum unfolded protein response (GO:0030968)	4	4.26	1.77E-02
Lymphocyte migration (GO:0072676)	4	4.26	1.77E-02
Cellular response to decreased oxygen levels (GO:0036294)	5	3.47	1.78E-02
Positive regulation of peptidyl-tyrosine phosphorylation (GO:0050731)	7	2.7	1.83E-02
Leukocyte chemotaxis (GO:0030595)	6	2.99	1.84E-02
Negative regulation of transcription by RNA polymerase II (GO:0000122)	23	1.67	1.86E-02
Negative regulation of cell-substrate adhesion (GO:0010812)	4	4.17	1.90E-02
Ameboidal-type cell migration (GO:0001667)	7	2.68	1.90E-02
Positive regulation of response to endoplasmic reticulum stress (GO:1905898)	3	5.75	1.91E-02
Positive regulation of leukocyte migration (GO:0002687)	6	2.96	1.92E-02
Leukocyte migration involved in inflammatory response (GO:0002523)	2	10.64	2.03E-02
Alditol phosphate metabolic process (GO:0052646)	2	10.64	2.03E-02
SMAD protein import into nucleus (GO:0007184)	2	10.64	2.03E-02
Leukotriene metabolic process (GO:0006691)	2	10.64	2.03E-02
Negative regulation of protein autophosphorylation (GO:0031953)	2	10.64	2.03E-02
Inhibition of cysteine-type endopeptidase activity involved in apoptotic process (GO:1990001)	2	10.64	2.03E-02
Basic amino acid transport (GO:0015802)	2	10.64	2.03E-02
Positive regulation of triglyceride biosynthetic process (GO:0010867)	2	10.64	2.03E-02
Regulation of skeletal muscle cell proliferation (GO:0014857)	2	10.64	2.03E-02
Pyrimidine-containing compound transmembrane transport (GO:0072531)	2	10.64	2.03E-02
Cellular carbohydrate metabolic process (GO:0044262)	7	2.64	2.04E-02
Negative regulation of cell migration (GO:0030336)	9	2.29	2.05E-02
Signal transduction by p53 class mediator (GO:0072331)	5	3.33	2.08E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of leukocyte chemotaxis (GO:0002690)	5	3.33	2.08E-02
Substantia nigra development (GO:0021762)	3	5.53	2.09E-02
Dendritic cell differentiation (GO:0097028)	3	5.53	2.09E-02
Negative regulation of production of molecular mediator of immune response (GO:0002701)	3	5.53	2.09E-02
Regulation of defense response (GO:0031347)	16	1.9	2.11E-02
Tumor necrosis factor-mediated signaling pathway (GO:0033209)	4	3.99	2.16E-02
Positive regulation of JAK-STAT cascade (GO:0046427)	4	3.99	2.16E-02
Regulation of stress-activated MAPK cascade (GO:0032872)	8	2.41	2.18E-02
Regulation of cellular component organization (GO:0051128)	53	1.37	2.21E-02
Regulation of stress-activated protein kinase signaling cascade (GO:0070302)	8	2.4	2.25E-02
Regulation of adaptive immune response (GO:0002819)	6	2.85	2.27E-02
Negative regulation of neuron death (GO:1901215)	7	2.58	2.28E-02
Positive regulation of intracellular signal transduction (GO:1902533)	24	1.63	2.29E-02
Blood vessel remodeling (GO:0001974)	3	5.32	2.29E-02
Regulation of T cell receptor signaling pathway (GO:0050856)	3	5.32	2.29E-02
Regulation of epithelial cell apoptotic process (GO:1904035)	4	3.91	2.30E-02
Regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043281)	8	2.38	2.33E-02
Positive regulation of chemotaxis (GO:0050921)	6	2.82	2.36E-02
Chemotaxis (GO:0006935)	14	1.9	2.38E-02
Biological_process (GO:0008150)	382	1.06	2.39E-02
Nucleobase catabolic process (GO:0046113)	2	9.58	2.40E-02
Negative regulation of T-helper cell differentiation (GO:0045623)	2	9.58	2.40E-02
T cell cytokine production (GO:0002369)	2	9.58	2.40E-02
Neutrophil homeostasis (GO:0001780)	2	9.58	2.40E-02
Export across plasma membrane (GO:0140115)	2	9.58	2.40E-02
Vacuolar proton-transporting V-type ATPpase complex assembly (GO:0070072)	2	9.58	2.40E-02
Proton-transporting V-type ATPase complex assembly (GO:0070070)	2	9.58	2.40E-02
Glial cell activation (GO:0061900)	2	9.58	2.40E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Positive regulation of execution phase of apoptosis (GO:1900119)	2	9.58	2.40E-02
Fibroblast migration (GO:0010761)	2	9.58	2.40E-02
Coronary vasculature morphogenesis (GO:0060977)	2	9.58	2.40E-02
Zymogen inhibition (GO:0097341)	2	9.58	2.40E-02
Inhibition of cysteine-type endopeptidase activity (GO:0097340)	2	9.58	2.40E-02
Negative regulation of cell fate commitment (GO:0010454)	2	9.58	2.40E-02
Phototransduction, visible light (GO:0007603)	2	9.58	2.40E-02
Regulation of ER to Golgi vesicle-mediated transport (GO:0060628)	2	9.58	2.40E-02
Negative regulation of T cell mediated immunity (GO:0002710)	2	9.58	2.40E-02
Positive regulation of cellular extravasation (GO:0002693)	2	9.58	2.40E-02
Regulation of intracellular pH (GO:0051453)	5	3.19	2.41E-02
Taxis (GO:0042330)	14	1.89	2.41E-02
Positive regulation of STAT cascade (GO:1904894)	4	3.83	2.44E-02
Cellular response to unfolded protein (GO:0034620)	4	3.83	2.44E-02
Regulation of protein kinase B signaling (GO:0051896)	6	2.79	2.46E-02
Negative regulation of protein processing (GO:0010955)	3	5.13	2.50E-02
Adipose tissue development (GO:0060612)	3	5.13	2.50E-02
Negative regulation of protein maturation (GO:1903318)	3	5.13	2.50E-02
Positive regulation of protein catabolic process (GO:0045732)	8	2.34	2.55E-02
Regulation of intrinsic apoptotic signaling pathway (GO:2001242)	7	2.5	2.61E-02
Negative regulation of intracellular signal transduction (GO:1902532)	16	1.79	2.64E-02
Negative regulation of immune effector process (GO:0002698)	5	3.11	2.64E-02
Negative regulation of neuron apoptotic process (GO:0043524)	6	2.74	2.66E-02
Negative regulation of cell-matrix adhesion (GO:0001953)	3	4.96	2.72E-02
Inorganic ion import across plasma membrane (GO:0099587)	3	4.96	2.72E-02
Regulation of protein autophosphorylation (GO:0031952)	3	4.96	2.72E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of establishment of planar polarity (GO:0090175)	3	4.96	2.72E-02
Inorganic cation import across plasma membrane (GO:0098659)	3	4.96	2.72E-02
G2/M transition of mitotic cell cycle (GO:0000086)	3	4.96	2.72E-02
Negative regulation of developmental process (GO:0051093)	23	1.64	2.78E-02
Cell-substrate adhesion (GO:0031589)	7	2.47	2.79E-02
Positive regulation of receptor recycling (GO:0001921)	2	8.71	2.80E-02
Positive regulation of lymphocyte chemotaxis (GO:0140131)	2	8.71	2.80E-02
Planar cell polarity pathway involved in neural tube closure (GO:0090179)	2	8.71	2.80E-02
Negative regulation of CD4-positive, alpha-beta T cell differentiation (GO:0043371)	2	8.71	2.80E-02
Regulation of cell proliferation (GO:0042127)	36	1.46	2.83E-02
Response to external stimulus (GO:0009605)	39	1.43	2.83E-02
Negative regulation of locomotion (GO:0040013)	10	2.19	2.85E-02
Response to abiotic stimulus (GO:0009628)	23	1.62	2.90E-02
Synaptic vesicle exocytosis (GO:0016079)	4	3.62	2.91E-02
Positive regulation of nitrogen compound metabolic process (GO:0051173)	66	1.3	2.92E-02
Carbohydrate metabolic process (GO:0005975)	15	1.87	2.93E-02
Negative regulation of endocytosis (GO:0045806)	3	4.79	2.94E-02
Positive regulation of cellular protein catabolic process (GO:1903364)	6	2.66	2.98E-02
Establishment of vesicle localization (GO:0051650)	7	2.43	2.98E-02
Regulation of protein metabolic process (GO:0051246)	62	1.31	2.99E-02
Cellular response to interferon-gamma (GO:0071346)	4	3.55	3.08E-02
Response to lipid (GO:0033993)	17	1.77	3.10E-02
Regulation of transport (GO:0051049)	40	1.41	3.10E-02
Pigmentation (GO:0043473)	5	2.96	3.16E-02
Leukocyte mediated immunity (GO:0002443)	7	2.4	3.18E-02
Movement of cell or subcellular component (GO:0006928)	31	1.48	3.18E-02
Cell cycle G2/M phase transition (GO:0044839)	3	4.64	3.18E-02
Positive regulation of gliogenesis (GO:0014015)	3	4.64	3.18E-02
Regulation of protein deacetylation (GO:0090311)	3	4.64	3.18E-02
Regulation of neutrophil chemotaxis (GO:0090022)	3	4.64	3.18E-02
Positive regulation of cellular metabolic process (GO:0031325)	68	1.29	3.19E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of antigen processing and presentation (GO:0002577)	2	7.98	3.23E-02
Mononuclear cell differentiation (GO:1903131)	2	7.98	3.23E-02
Phospholipase C-activating dopamine receptor signaling pathway (GO:0060158)	2	7.98	3.23E-02
Negative regulation of focal adhesion assembly (GO:0051895)	2	7.98	3.23E-02
Vesicle fusion to plasma membrane (GO:0099500)	2	7.98	3.23E-02
Regulation of establishment of planar polarity involved in neural tube closure (GO:0090178)	2	7.98	3.23E-02
Positive regulation of T cell receptor signaling pathway (GO:0050862)	2	7.98	3.23E-02
Synaptic vesicle fusion to presynaptic active zone membrane (GO:0031629)	2	7.98	3.23E-02
Monocyte differentiation (GO:0030224)	2	7.98	3.23E-02
Response to muscle activity (GO:0014850)	2	7.98	3.23E-02
Granulocyte chemotaxis (GO:0071621)	4	3.48	3.25E-02
Cellular response to oxygen levels (GO:0071453)	5	2.92	3.30E-02
Positive regulation of stress-activated MAPK cascade (GO:0032874)	6	2.59	3.32E-02
Maintenance of location (GO:0051235)	7	2.36	3.38E-02
Lipid metabolic process (GO:0006629)	29	1.5	3.42E-02
Positive regulation of protein modification process (GO:0031401)	29	1.5	3.42E-02
Negative regulation of endothelial cell proliferation (GO:0001937)	3	4.49	3.43E-02
Dopamine receptor signaling pathway (GO:0007212)	3	4.49	3.43E-02
DNA damage response, signal transduction by p53 class mediator (GO:0030330)	3	4.49	3.43E-02
Positive regulation of glucose import (GO:0046326)	3	4.49	3.43E-02
Positive regulation of stress-activated protein kinase signaling cascade (GO:0070304)	6	2.57	3.44E-02
Vesicle-mediated transport (GO:0016192)	29	1.5	3.45E-02
Cellular lipid metabolic process (GO:0044255)	24	1.57	3.47E-02
Leukocyte activation involved in immune response (GO:0002366)	7	2.34	3.49E-02
Response to drug (GO:0042493)	17	1.7	3.54E-02
Response to mechanical stimulus (GO:0009612)	6	2.54	3.57E-02
Central nervous system neuron development (GO:0021954)	4	3.36	3.61E-02
Negative regulation of CD4-positive, alpha-beta T cell activation (GO:2000515)	2	7.37	3.67E-02
Negative regulation of cell division (GO:0051782)	2	7.37	3.67E-02
Maintenance of organelle location (GO:0051657)	2	7.37	3.67E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Proton-transporting two-sector atpase complex assembly (GO:0070071)	2	7.37	3.67E-02
Regulation of developmental pigmentation (GO:0048070)	2	7.37	3.67E-02
Negative regulation of lipid storage (GO:0010888)	2	7.37	3.67E-02
Ferric iron transport (GO:0015682)	2	7.37	3.67E-02
Venous blood vessel development (GO:0060841)	2	7.37	3.67E-02
Positive regulation of T-helper 1 type immune response (GO:0002827)	2	7.37	3.67E-02
Negative regulation of adherens junction organization (GO:1903392)	2	7.37	3.67E-02
Trivalent inorganic cation transport (GO:0072512)	2	7.37	3.67E-02
Neural crest cell migration (GO:0001755)	3	4.35	3.69E-02
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (GO:0002460)	6	2.52	3.69E-02
Vesicle localization (GO:0051648)	7	2.31	3.71E-02
Regulation of peptide secretion (GO:0002791)	12	1.96	3.72E-02
Maintenance of location in cell (GO:0051651)	5	2.82	3.74E-02
Positive regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043280)	5	2.82	3.74E-02
Metal ion homeostasis (GO:0055065)	15	1.74	3.75E-02
Positive regulation of macromolecule metabolic process (GO:0010604)	67	1.28	3.78E-02
Regulation of cellular protein metabolic process (GO:0032268)	58	1.31	3.78E-02
Cardiac septum morphogenesis (GO:0060411)	4	3.3	3.80E-02
Cellular iron ion homeostasis (GO:0006879)	4	3.3	3.80E-02
Leukocyte homeostasis (GO:0001776)	4	3.3	3.80E-02
Regulation of response to endoplasmic reticulum stress (GO:1905897)	4	3.3	3.80E-02
Apoptotic signaling pathway (GO:0097190)	10	2	3.86E-02
Regulation of cell-matrix adhesion (GO:0001952)	5	2.78	3.89E-02
Lipid homeostasis (GO:0055088)	5	2.78	3.89E-02
Lung alveolus development (GO:0048286)	3	4.23	3.95E-02
Developmental pigmentation (GO:0048066)	3	4.23	3.95E-02
Positive regulation of protein tyrosine kinase activity (GO:0061098)	3	4.23	3.95E-02
Regulation of gliogenesis (GO:0014013)	4	3.25	4.00E-02
Outflow tract morphogenesis (GO:0003151)	4	3.25	4.00E-02
Negative regulation of cell motility (GO:2000146)	9	2.19	4.03E-02
Negative regulation of T cell antigen processing and presentation (GO:0002626)	1	47.9	4.05E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of T cell antigen processing and presentation (GO:0002625)	1	47.9	4.05E-02
Negative regulation of asymmetric cell division (GO:0045769)	1	47.9	4.05E-02
Positive regulation of leukocyte tethering or rolling (GO:1903238)	1	47.9	4.05E-02
Toll-like receptor 10 signaling pathway (GO:0034166)	1	47.9	4.05E-02
Establishment or maintenance of cell polarity regulating cell shape (GO:0071963)	1	47.9	4.05E-02
Spleen trabecula formation (GO:0060345)	1	47.9	4.05E-02
Cellular cadmium ion homeostasis (GO:0006876)	1	47.9	4.05E-02
Purine nucleoside bisphosphate catabolic process (GO:0034034)	1	47.9	4.05E-02
Ribonucleoside bisphosphate catabolic process (GO:0034031)	1	47.9	4.05E-02
T cell antigen processing and presentation (GO:0002457)	1	47.9	4.05E-02
Cell proliferation in bone marrow (GO:0071838)	1	47.9	4.05E-02
Negative regulation of vasculogenesis (GO:2001213)	1	47.9	4.05E-02
Negative regulation of eosinophil activation (GO:1902567)	1	47.9	4.05E-02
Negative regulation of CD8-positive, alpha-beta T cell activation (GO:2001186)	1	47.9	4.05E-02
MHC class II biosynthetic process (GO:0045342)	1	47.9	4.05E-02
VEGF-activated neuropilin signaling pathway (GO:0038190)	1	47.9	4.05E-02
Positive regulation of B cell chemotaxis (GO:2000538)	1	47.9	4.05E-02
Regulation of B cell chemotaxis (GO:2000537)	1	47.9	4.05E-02
VEGF-activated neuropilin signaling pathway involved in axon guidance (GO:1902378)	1	47.9	4.05E-02
Interleukin-11-mediated signaling pathway (GO:0038154)	1	47.9	4.05E-02
Positive regulation of retinal ganglion cell axon guidance (GO:1902336)	1	47.9	4.05E-02
Regulation of copper ion transmembrane transport (GO:1902311)	1	47.9	4.05E-02
Positive regulation of hematopoietic stem cell migration (GO:2000473)	1	47.9	4.05E-02
Regulation of hematopoietic stem cell migration (GO:2000471)	1	47.9	4.05E-02
Positive regulation of astrocyte chemotaxis (GO:2000464)	1	47.9	4.05E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Negative regulation of mast cell cytokine production (GO:0032764)	1	47.9	4.05E-02
Positive regulation of monocyte extravasation (GO:2000439)	1	47.9	4.05E-02
Positive regulation of eosinophil chemotaxis (GO:2000424)	1	47.9	4.05E-02
Regulation of eosinophil chemotaxis (GO:2000422)	1	47.9	4.05E-02
Positive regulation of hepatocyte growth factor receptor signaling pathway (GO:1902204)	1	47.9	4.05E-02
Diaphragm contraction (GO:0002086)	1	47.9	4.05E-02
Leukotriene D4 biosynthetic process (GO:1901750)	1	47.9	4.05E-02
Leukotriene D4 metabolic process (GO:1901748)	1	47.9	4.05E-02
Regulation of glycogen catabolic process (GO:0005981)	1	47.9	4.05E-02
Ovarian follicle rupture (GO:0001543)	1	47.9	4.05E-02
Interleukin-3 production (GO:0032632)	1	47.9	4.05E-02
Peptidyl-serine trans-autophosphorylation (GO:1990579)	1	47.9	4.05E-02
Negative regulation of secretion of lysosomal enzymes (GO:0090341)	1	47.9	4.05E-02
Positive regulation of peptidyl-cysteine S-nitrosylation (GO:2000170)	1	47.9	4.05E-02
Rab protein signal transduction (GO:0032482)	1	47.9	4.05E-02
Histone H3-K9 acetylation (GO:0043970)	1	47.9	4.05E-02
Positive regulation of immune complex clearance by monocytes and macrophages (GO:0090265)	1	47.9	4.05E-02
Regulation of immune complex clearance by monocytes and macrophages (GO:0090264)	1	47.9	4.05E-02
Cardiac endothelial to mesenchymal transition (GO:0140074)	1	47.9	4.05E-02
Regulation of ER to Golgi vesicle-mediated transport by GTP hydrolysis (GO:0090113)	1	47.9	4.05E-02
Positive regulation of calcium ion import across plasma membrane (GO:1905665)	1	47.9	4.05E-02
Peptidyl-histidine dephosphorylation (GO:0035971)	1	47.9	4.05E-02
Negative regulation of retrograde trans-synaptic signaling by neuropeptide (GO:1905433)	1	47.9	4.05E-02
Regulation of retrograde trans-synaptic signaling by neuropeptide (GO:1905432)	1	47.9	4.05E-02
Innate vocalization behavior (GO:0098582)	1	47.9	4.05E-02
Positive regulation of amyloid precursor protein biosynthetic process (GO:0042986)	1	47.9	4.05E-02
Protein trans-autophosphorylation (GO:0036290)	1	47.9	4.05E-02
D-serine transport (GO:0042942)	1	47.9	4.05E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
D-alanine transport (GO:0042941)	1	47.9	4.05E-02
mRNA splicing via endonucleolytic cleavage and ligation involved in unfolded protein response (GO:0030969)	1	47.9	4.05E-02
Negative regulation of G1/S transition of mitotic cell cycle by negative regulation of transcription from RNA polymerase II promoter (GO:1900477)	1	47.9	4.05E-02
T-helper 17 cell chemotaxis (GO:0035705)	1	47.9	4.05E-02
Helper T cell chemotaxis (GO:0035704)	1	47.9	4.05E-02
Negative regulation of eosinophil degranulation (GO:0043310)	1	47.9	4.05E-02
Pyridoxal 5'-phosphate salvage (GO:0009443)	1	47.9	4.05E-02
Negative regulation of short-term neuronal synaptic plasticity (GO:0048174)	1	47.9	4.05E-02
mRNA endonucleolytic cleavage involved in unfolded protein response (GO:0070055)	1	47.9	4.05E-02
mRNA splicing, via endonucleolytic cleavage and ligation (GO:0070054)	1	47.9	4.05E-02
Regulation of granulosa cell apoptotic process (GO:1904708)	1	47.9	4.05E-02
Tricarboxylic acid transmembrane transport (GO:0035674)	1	47.9	4.05E-02
Positive regulation of protein localization to phagocytic vesicle (GO:1905171)	1	47.9	4.05E-02
Regulation of protein localization to phagocytic vesicle (GO:1905169)	1	47.9	4.05E-02
Regulation of transcription involved in G2/M transition of mitotic cell cycle (GO:0000117)	1	47.9	4.05E-02
Negative regulation of astrocyte activation (GO:0061889)	1	47.9	4.05E-02
Vacuolar sequestering (GO:0043181)	1	47.9	4.05E-02
Maltose metabolic process (GO:0000023)	1	47.9	4.05E-02
Otic placode development (GO:1905040)	1	47.9	4.05E-02
Coenzyme A catabolic process (GO:0015938)	1	47.9	4.05E-02
Pyrimidine nucleoside transport (GO:0015864)	1	47.9	4.05E-02
Ferrous iron export across plasma membrane (GO:1903988)	1	47.9	4.05E-02
Negative regulation of microglial cell activation (GO:1903979)	1	47.9	4.05E-02
UDP-glucose transmembrane transport (GO:0015786)	1	47.9	4.05E-02
Nitrite transport (GO:0015707)	1	47.9	4.05E-02
Coreceptor-mediated virion attachment to host cell (GO:0046814)	1	47.9	4.05E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Negative regulation of antigen processing and presentation of endogenous peptide antigen via MHC class I (GO:1904283)	1	47.9	4.05E-02
Regulation of antigen processing and presentation of endogenous peptide antigen via MHC class I (GO:1904282)	1	47.9	4.05E-02
Positive regulation of glutathione biosynthetic process (GO:1903788)	1	47.9	4.05E-02
Regulation of glutathione biosynthetic process (GO:1903786)	1	47.9	4.05E-02
Cerebellum vasculature morphogenesis (GO:0061301)	1	47.9	4.05E-02
Regulation of COPII vesicle coating (GO:0003400)	1	47.9	4.05E-02
Negative regulation of gap junction assembly (GO:1903597)	1	47.9	4.05E-02
Propionate metabolic process, methylcitrate cycle (GO:0019679)	1	47.9	4.05E-02
Iron cation export (GO:1903414)	1	47.9	4.05E-02
Protein localization to early endosome (GO:1902946)	1	47.9	4.05E-02
Nucleoside bisphosphate catabolic process (GO:0033869)	1	47.9	4.05E-02
Propionate metabolic process (GO:0019541)	1	47.9	4.05E-02
Fatty acid derivative metabolic process (GO:1901568)	5	2.75	4.05E-02
Coagulation (GO:0050817)	6	2.46	4.09E-02
Blood coagulation (GO:0007596)	6	2.46	4.09E-02
Negative regulation of immune system process (GO:0002683)	12	1.89	4.09E-02
Regulation of developmental process (GO:0050793)	51	1.33	4.10E-02
Immune effector process (GO:0002252)	14	1.82	4.10E-02
Branching involved in salivary gland morphogenesis (GO:0060445)	2	6.84	4.14E-02
Respiratory system process (GO:0003016)	2	6.84	4.14E-02
Cell volume homeostasis (GO:0006884)	2	6.84	4.14E-02
G-protein coupled acetylcholine receptor signaling pathway (GO:0007213)	2	6.84	4.14E-02
DNA replication-independent nucleosome assembly (GO:0006336)	2	6.84	4.14E-02
Defense response to protozoan (GO:0042832)	2	6.84	4.14E-02
Leukocyte adhesion to vascular endothelial cell (GO:0061756)	2	6.84	4.14E-02
Positive regulation of histone deacetylation (GO:0031065)	2	6.84	4.14E-02
Regulation of triglyceride biosynthetic process (GO:0010866)	2	6.84	4.14E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Negative regulation of alpha-beta T cell differentiation (GO:0046639)	2	6.84	4.14E-02
Negative regulation of binding (GO:0051100)	6	2.44	4.22E-02
Negative regulation of secretion by cell (GO:1903531)	6	2.44	4.22E-02
Regulation of neutrophil migration (GO:1902622)	3	4.11	4.23E-02
Calcium ion transport (GO:0006816)	9	2.12	4.32E-02
Hemostasis (GO:0007599)	6	2.42	4.36E-02
Regulation of protein phosphorylation (GO:0001932)	34	1.42	4.37E-02
Negative regulation of multicellular organismal process (GO:0051241)	25	1.5	4.44E-02
Cell adhesion (GO:0007155)	20	1.58	4.50E-02
Cellular glucose homeostasis (GO:0001678)	3	3.99	4.51E-02
Regulation of endothelial cell apoptotic process (GO:2000351)	3	3.99	4.51E-02
Cellular response to ammonium ion (GO:0071242)	3	3.99	4.51E-02
Lymphocyte chemotaxis (GO:0048247)	3	3.99	4.51E-02
Negative regulation of DNA binding (GO:0043392)	3	3.99	4.51E-02
Positive regulation of cell killing (GO:0031343)	3	3.99	4.51E-02
Positive regulation of glucose transport (GO:0010828)	3	3.99	4.51E-02
Post-embryonic development (GO:0009791)	5	2.66	4.55E-02
Lymphocyte activation (GO:0046649)	12	1.83	4.58E-02
Regulation of protein tyrosine kinase activity (GO:0061097)	4	3.09	4.61E-02
Regulation of protein maturation (GO:1903317)	4	3.09	4.61E-02
Glycoprotein catabolic process (GO:0006516)	2	6.39	4.63E-02
Response to protozoan (GO:0001562)	2	6.39	4.63E-02
Regulation of epithelial tube formation (GO:1905276)	2	6.39	4.63E-02
Antigen processing and presentation, exogenous lipid antigen via MHC class Ib (GO:0048007)	2	6.39	4.63E-02
Antigen processing and presentation of lipid antigen via MHC class Ib (GO:0048003)	2	6.39	4.63E-02
DNA replication-independent nucleosome organization (GO:0034724)	2	6.39	4.63E-02
Kidney vasculature development (GO:0061440)	2	6.39	4.63E-02
Renal system vasculature development (GO:0061437)	2	6.39	4.63E-02
Regulation of T-helper 1 type immune response (GO:0002825)	2	6.39	4.63E-02
Peptidyl-tyrosine phosphorylation (GO:0018108)	6	2.38	4.65E-02
Negative regulation of MAPK cascade (GO:0043409)	6	2.36	4.80E-02
Regulation of leukocyte mediated immunity (GO:0002703)	6	2.36	4.80E-02
Sprouting angiogenesis (GO:0002040)	3	3.88	4.81E-02
Positive regulation of wound healing (GO:0090303)	3	3.88	4.81E-02

Table A.2 continued

GO biological process complete	#genes	FE	*P*-value
Regulation of transcription from RNA polymerase II promoter in response to stress (GO:0043618)	3	3.88	4.81E-02
Oligosaccharide metabolic process (GO:0009311)	3	3.88	4.81E-02
Positive regulation of response to biotic stimulus (GO:0002833)	3	3.88	4.81E-02
Positive regulation of cysteine-type endopeptidase activity (GO:2001056)	5	2.6	4.90E-02
Cellular response to leukemia inhibitory factor (GO:1990830)	5	2.6	4.90E-02
Response to leukemia inhibitory factor (GO:1990823)	5	2.6	4.90E-02
Cellular response to metal ion (GO:0071248)	5	2.6	4.90E-02

#genes: number of genes associated with the GO term in the list of differentially expressed genes (DEG, q-value < 0.05)
FE: Fold Enrichment